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STATE OF MICHIGAN
DEPARTMENT OF LICENSING AND REGULATORY AFFAIRS
BUREAU OF HEALTH CARE SERVICES
MICHIGAN MEDICAL MARIHUANA REVIEW PANEL

Dear, Director of LARA and the Michigan Medical Marihuana Review Panel,

It is with great importance that Four Freedoms submits the enclosed three packets of evidentiary documentation in response to the decision of the Michigan Medical Marijuana Review Panel to deny PTSd as a qualifying condition to the Michigan Medical Marijuana Act of 2008.

With respect to the process and individual members of the Michigan Medical Marijuana Review Panel, understanding panel's decision was based on a lack of scientific evidence and insufficient antidotal evidence.

Respectfully request the following three packages of information be presented to the Michigan Medical Marijuana Review Panel, in support of the recommendation to include PTSd in the MMMA of 2008.

Packet 1) 13 research papers supporting the use of cannabis to treat symptoms of PTSd.

Packet 2) 7 research papers supporting the use of cannabis as harm reduction.

Packet 3) New Mexico Medical Cannabis Program Advisory Board final report 7 Nov 2012, media reports relevant to issue from New Mexico, and additional antidotal evidence.

I am extremely grateful for the open and honest dialogue among the members of the panel. As a Veteran, all I can ask is that this issue be taken seriously, it is obvious from comments made by the MMMRP members, December 14, 2012 that members of the panel are focused on patient care and for that I am eternally grateful.

Additional information provided with the assistance of the following groups, Veterans for Medical Marijuana Access, The Drug Policy Alliance and Patients Out of Time.

Sincerely yours,

John Evans

Four Freedoms

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Packet #1, Research papers supporting the use of cannabis to treat symptoms of PTSd.

MEDICAL CANNABIS AS TREATMENT FOR CHRONIC COMBAT PTSD

Promising Results in an Open Pilot Study

Mordechai Mashiah, MD, MHA.
Deputy Director,
Abarbanel Mental Hospital, Israel

Patients Out of Time Conference,
Tucson, Arizona April 28, 2012

"Yehuda Abarbanel"
Mental Health Center

INTRODUCTION

- I am the Deputy Director of the Abarbanel Mental Hospital - the largest mental hospital in Israel with 300+ beds
- For the past 3 years the licensing of the cannabis treatment program for participants suffering from PTSD has been under my charge in Israel.
- Currently due to Ministry of Defense restrictions as to combat-related PTSD out of the ~8,000 total patients treated with cannabis in Israel there are only ~200 participants receiving cannabis to treat chronic PTSD.

DSM-IV-TR CRITERIA FOR PTSD

A disorder based on several criterion experienced over time.

CRITERION
A

STRESSOR

- The person has been exposed to a traumatic event in which both of the following have been present:
- The person has experienced, witnessed, or been confronted with an event or events that involve actual or threatened death or serious injury, or a threat to the physical integrity of oneself or others.
- The person's response involved intense fear, helplessness, or horror.

A
B
C
D
E
F

CRITERION B INTRUSIVE RECOLLECTION

The traumatic event is persistently re-experienced in at least one of the following ways:

1. Recurrent and intrusive distressing recollections of the event, including images, thoughts, or perceptions.
2. Recurrent distressing dreams of the event.
3. Acting or feeling as if the traumatic event were recurring (includes a sense of reliving the experience, illusions, hallucinations, and dissociative flashback episodes, including those that occur upon awakening or when intoxicated).
4. Intense psychological distress at exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event.
5. Physiologic reactivity upon exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event

CRITERION C

AVOIDANT/NUMBING

Persistent avoidance of stimuli associated with the trauma and numbing of general responsiveness (not present before the trauma), as indicated by at least three of the following:

1. Efforts to avoid thoughts, feelings, or conversations associated with the trauma
2. Efforts to avoid activities, places, or people that arouse recollections of the trauma
3. Inability to recall an important aspect of the trauma
4. Markedly diminished interest or participation in significant activities
5. Feeling of detachment or estrangement from others
6. Restricted range of affect (e.g., unable to have loving feelings)
7. Sense of foreshortened future (e.g., does not expect to have a career, marriage, children, or a normal life span)

A

B

C

D

E

F

CRITERION
D

HYPER AROUSAL

Persistent symptoms of increasing arousal (not present before the trauma), indicated by at least two of the following:

1. Difficulty falling or staying asleep
2. Irritability or outbursts of anger
3. Difficulty concentrating
4. Hyper-vigilance
5. Exaggerated startle response

A
B
C
D
E
F

CRITERION
E

DURATION

Duration of the disturbance (symptoms in B, C, and D) is more than one month.

A
B
C
D
E
F

CRITERION F FUNCTIONAL SIGNIFICANCE

The disturbance causes clinically significant distress or impairment in social, occupational, or other important areas of functioning.

A

B

C

D

E

F

DSM-IV ***PTSD***

Specify if:

Acute:
If duration of symptoms is less than three months

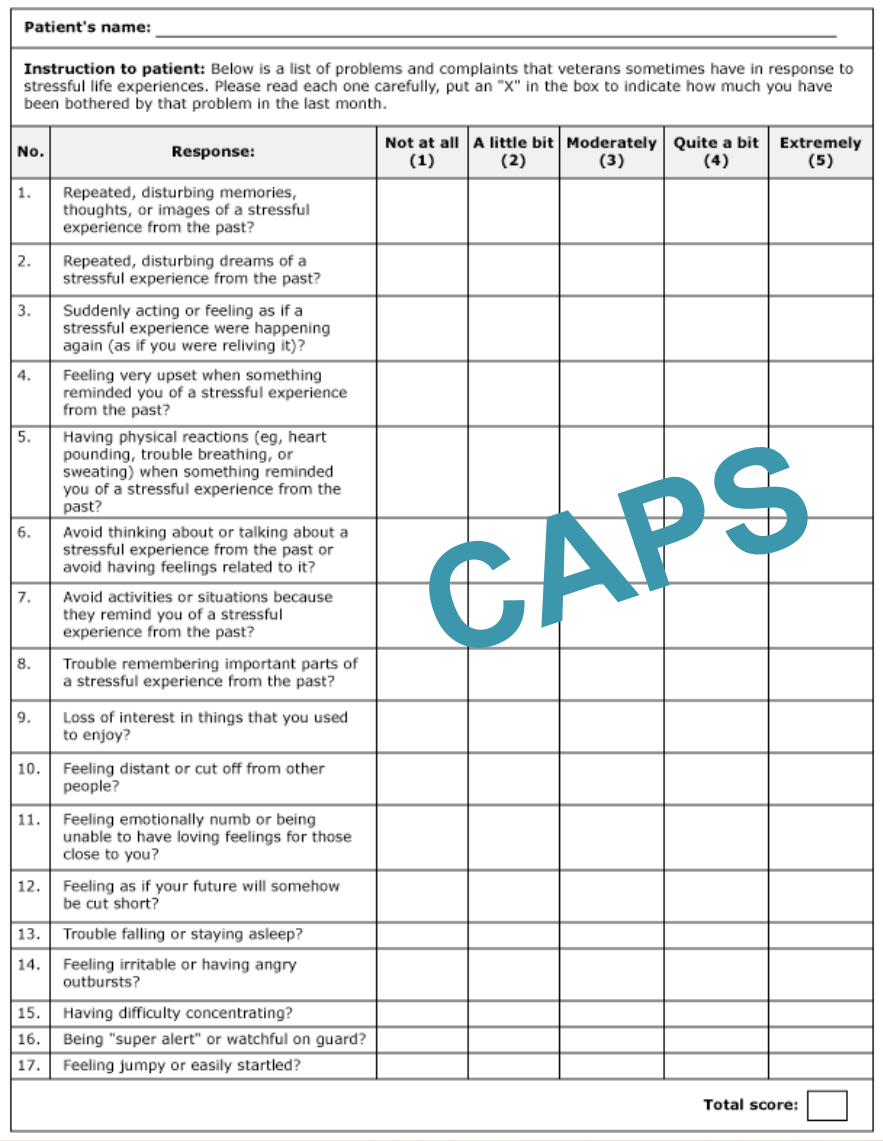
Chronic:
If duration of symptoms is three months or more

Specify if:

With or Without delay onset:
Onset of symptoms at least six months after the stressor

CLINICAL ADMINISTERED PTSD (CAPS)

- This is an initial self-report symptom checklist.
- It covers all kinds of PTSD symptoms.
- The self-report checklist is used by the clinician to guide the interview for the assessment.
- CAPS has become the standard of PTSD assessment because it gives clinicians the ability to focus on the most effective areas of treatment.



Patient's name: _____

Instruction to patient: Below is a list of problems and complaints that veterans sometimes have in response to stressful life experiences. Please read each one carefully, put an "X" in the box to indicate how much you have been bothered by that problem in the last month.

No.	Response:	Not at all (1)	A little bit (2)	Moderately (3)	Quite a bit (4)	Extremely (5)
1.	Repeated, disturbing memories, thoughts, or images of a stressful experience from the past?					
2.	Repeated, disturbing dreams of a stressful experience from the past?					
3.	Suddenly acting or feeling as if a stressful experience were happening again (as if you were reliving it)?					
4.	Feeling very upset when something reminded you of a stressful experience from the past?					
5.	Having physical reactions (eg, heart pounding, trouble breathing, or sweating) when something reminded you of a stressful experience from the past?					
6.	Avoid thinking about or talking about a stressful experience from the past or avoid having feelings related to it?					
7.	Avoid activities or situations because they remind you of a stressful experience from the past?					
8.	Trouble remembering important parts of a stressful experience from the past?					
9.	Loss of interest in things that you used to enjoy?					
10.	Feeling distant or cut off from other people?					
11.	Feeling emotionally numb or being unable to have loving feelings for those close to you?					
12.	Feeling as if your future will somehow be cut short?					
13.	Trouble falling or staying asleep?					
14.	Feeling irritable or having angry outbursts?					
15.	Having difficulty concentrating?					
16.	Being "super alert" or watchful on guard?					
17.	Feeling jumpy or easily startled?					

Total score: ☐

PTSD IN ISRAEL

Baseline PTSD in this study:

- Study symptom severity (CAPS score) was high : (97.7 +/- 13.3)
- Israel is a stressful and dense place with many wars and terrorist attacks. As a result we have many victims of PTSD. I believe the baseline for PTSD is probably higher in general in Israel.
- These were already well established chronic and severe PTSD sufferers.

OUR OBJECTIVE: REMISSION

The efficacy of currently available medications in the treatment of chronic combat post-traumatic stress disorder (PTSD) is variable, with some patients not achieving remission.

- This open pilot study was designed to test the effects of smoked cannabis on symptoms of chronic combat PTSD.

METHODOLOGY (2008)

Enrollment Profile

A small number (N=29) of Israeli male, combat veterans, diagnosed with PTSD

- by the Israel Defense Force PTSD Unit

or

- by the Ministry of Defense Rehabilitation Division.

Assessments

- Assessments included PTSD symptom severity using the (CAPS) interview
- a self-assessment of quality of life (QOL) (part of CAPS)
- a clinician-assessment of clinical improvement.

METHODOLOGY (2008)

- **How our participants used Cannabis?**

Smoked medical indica cannabis of roughly 23% THC and less than 1% CBD was dispensed to the subjects:

- at an amount of no more than 100 grams per month (based on their license's limit and set at a high level to thwart undue distress)
- Cannabis was added to subjects' standing treatment
- Subjects were instructed to smoke the cannabis daily at times, frequencies and amounts of their own choosing until they felt relaxed.

- **Follow up: what we learned?**

CAPS assessments were conducted by the Patient's Psychiatrist:

- At onset, and then at an average of:
- **4.3** months, (+/- 3.3 months),
- **7.6** months (+/- 2.7 months),
- **11.3** months (+/- 2.9 months).

MEDICAL CANNABIS

PRELIMINARY RESULTS

- 29 started the study.
- 26 completed the second CAPS
- 25 completed the third CAPS
- 10 completed the final CAPS

PRELIMINARY RESULTS

What happened following cannabis use?

- On average, symptom reduction in the remaining 26 subjects was seen in the second CAPS assessment:

CAPS Assessment	Average Duration since last CAPS in Months	Average CAPS Global Severity Score*
Baseline	-	97.7 ± 13.3
2	4.3 ± 3.3	60.3 ± 20.1
3	7.6 ± 2.7	57.0 ± 20.6
4	11.3 ± 2.9	53.7 ± 18.3

* CAPS Global Severity score of 50 is diagnostic cut-off for moderate PTSD

CONCLUSIONS

- Use of medical cannabis was associated with a reduction in PTSD symptoms in this open-label pilot study.
- Larger studies using randomized, double-blind methodology are needed to demonstrate a causal relationship.
- Results show that after: 4.3, 7.6 and 11.3 months, patients still had moderate to severe PTSD.

FUTURE RESEARCH

- Additional areas of study include:
- Identifying the active ingredients in cannabis that help with PTSD
- Establishing appropriate dose and duration of treatment
- Determining how cannabis reduces the need for other medications
- Clarifying risks of abuse and other legal aspects of medical cannabis use.

WHAT WE ARE DOING TODAY

I believe cannabis can be an effective part of a holistic care treatment in our clinics

- ✓ We have begun planning a new double-blind randomized controlled trial based on the requirements put forth by our Ministry of Rehabilitation (part of our Ministry of Defense) mandating: clinical trials to establish causality between cannabis as treatment and PTSD.
- ✓ We have begun planning a retrospective study to assess the reduction of medication cost and use.

QUESTIONS / COMMENTS?

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Thank you very much and Shalom

Review

Drug Testing and Analysis

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(www.drugtestinganalysis.com) DOI 10.1002/dta.1377

Mitigation of post-traumatic stress symptoms by *Cannabis* resin: A review of the clinical and neurobiological evidence

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It is known from clinical studies that some patients attempt to cope with the symptoms of post-traumatic stress disorder (PTSD) by using recreational drugs. This review presents a case report of a 19-year-old male patient with a spectrum of severe PTSD symptoms, such as intense flashbacks, panic attacks, and self-mutilation, who discovered that some of his major symptoms were dramatically reduced by smoking cannabis resin. The major part of this review is concerned with the clinical and preclinical neurobiological evidence in order to offer a potential explanation of these effects on symptom reduction in PTSD. This review shows that recent studies provided supporting evidence that PTSD patients may be able to cope with their symptoms by using cannabis products. Cannabis may dampen the strength or emotional impact of traumatic memories through synergistic mechanisms that might make it easier for people with PTSD to rest or sleep and to feel less anxious and less involved with flashback memories. The presence of endocannabinoid signalling systems within stress-sensitive nuclei of the hypothalamus, as well as upstream limbic structures (amygdala), point to the significance of this system for the regulation of neuroendocrine and behavioural responses to stress. Evidence is increasingly accumulating that cannabinoids might play a role in fear extinction and antidepressive effects. It is concluded that further studies are warranted in order to evaluate the therapeutic potential of cannabinoids in PTSD.

Keywords: cannabis resin; THC; cannabinoids; posttraumatic stress disorder (PTSD); psychopharmacology; endocannabinoid system

Introduction: PTSD and cannabinoids

Clinical evidence obtained from clinical studies shows that people suffering from post-traumatic stress disorder (PTSD) may use recreational drugs to cope with their symptoms.[1] Some specific psychopharmacological effects of cannabis, such as sedation, relaxation, reduction of anxiety and sleep-induction, may explain its use as an attempt to cope with some PTSD symptoms.[1–4] Cannabis products have been used medicinally in Asia and Europe as sedatives or calmatives, including the Western medical tradition up to the early twentieth century.[5] Cannabis was also listed in the United States Pharmacopeia and Formulary until its removal in 1941.[6] Many patients with PTSD may actually cope with their symptoms in this way, as stated by the discoverer of Δ^9-tetrahydrocannabinol (THC)[7] who reported that use of cannabis led to improved sleep, significant reduction of nightmares and sleep interruption.[8] Marijuana use has emerged as one of the most commonly used illicit substance in treatment-seeking adolescents[9] and it has been suggested that cannabis use is significantly more common among adolescents with PTSD than in those without this condition.[10] More recently, some studies and surveys found even stronger evidence that cannabinoids are used in a larger population of patients with PTSD for coping with their symptoms.[11–15] Bonn-Miller *et al.*[11] examined cannabis use in PTSD patients and the interaction of PTSD-related sleep disorders, symptom severity, and motivations for use. These authors found a strong correlation between the severity of PTSD-related sleep disturbances and the amount of cannabis use. These results have to be taken with caution, because the evidence for sleep-enhancing effects of cannabis resin and marijuana is equivocal (see subsection on sleep-enhancing effects). An effect on sleep may also result from the decrease of symptoms of over-arousal, which would be consistent with findings involving self-medicating populations of PTSD patients.[1,2,13] Bujarski *et al.*[13] also studied alternative motives for use and demonstrated that in adolescents with PTSD the coping motive was the primary cause for use and all other motives examined, i.e. 'social', 'enhancement' or 'conformity', were close to zero. These results were limited to a population of PTSD patients seeking treatment for substance abuse, and therefore, generalizability seems limited. Another study reported a strong correlation between PTSD symptom severity and the amount of cannabis use[14,15] and discussed the self-medication hypothesis as a possible

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explanation.[14] An additional interesting finding was that the starting point of using cannabis correlated with the onset of PTSD symptoms in more than half of the sample.[15] These authors speculated that cannabis use was used to help alleviate aversive mood states, but this hypothesis was not confirmed by another more limited and smaller study carried out by Bujarski *et al.*[13]

Another specific study involving combat veterans who displayed severe PTSD pathology examined the symptom clusters individually by *post hoc* analyses in a correlation analysis of psychopathological relapse causes four months after treatment. Main causal factors for relapse were found in avoidance and numbing symptom clusters. A further more specific analysis suggested a unique predictive effect for the PTSD numbing symptom cluster, but not for the avoidance symptom cluster, which indicated that primarily numbing symptoms may be a risk factor for increased cannabis use in patients with PTSD.[12] Another interesting finding of this study was the fact that changes in PTSD symptom severity were not incrementally predictive of any non-cannabis substance use and the authors discussed this in context of limitations of their study. However, this may point towards some specific effects of cannabis on some major symptoms of PTSD. It has to be mentioned that all these studies used a cross-sectional methodology and so their results cannot solve questions regarding temporal and causal directionality underlying the observed effects.

Most of the studies mentioned here appear to have implications for clinical practice. It is implicated that more attention has to be paid to the comorbidity of cannabis users and the background of their motives for use, especially in those with cannabis dependency syndromes. Their treatment has to pay more attention to these comorbidities and at best offer a combined treatment for addictive behaviour and PTSD.

Recently, a study protocol which was introduced for consideration by the US regulatory agencies for the study of cannabis resin for the treatment of PTSD was rejected, but permits were provided for the use of medical marijuana in PTSD in two states of the USA (Delaware and New Mexico).[16]

There is some robust evidence from clinical and preclinical studies that the endocannabinoid system (eCB) may be involved in the pathogenesis of several psychopathological symptoms.[17,18] It was recently concluded that the endocannabinoid system is implicated in homoeostatic cortical excitation and inhibition as well as in emotional homoeostasis.[19] There is also growing evidence for antidepressant and anxiolytic effects of the major cannabis ingredients THC and cannabidiol (CBD).[20] This review aims to consider whether neurochemical alterations as well as changes in neurobiological functioning might underlie these effects.

A clinical case report

From about the age four, the patient was a victim of long-time sadistic sexual abuse by his father and paternal uncle, which continued until age 15 when he attempted to commit suicide for the second time following the first suicide attempt two years earlier. Since then, this patient has been closely followed in outpatient psychiatric clinics. Because he was not diagnosed with PTSD at first, he did not receive treatments specific to PTSD for years. We first saw the patient in April 2004 when he was admitted to an acute psychiatric ward of our department for safety and stabilization during a crisis with severe, uncontrolled flashbacks, panic attacks, and impulses for self-mutilation. His physical examination was without abnormalities and drug testing was negative. Usually, he was treated during/after these states with sublingual lorazepam up to 10 mg/day. In a typical and severe flashback episode, this patient appeared in a dissociative state with complete loss of self-control. He would cry intensely, fall down, thrash about uncontrollably, and did not appear to have any cognitive or emotional control over re-experiencing past trauma. Immediately after such episodes, the patient would experience severe urges for self-mutilation. Such urges had resulted in severe self-injury in the past (mainly lacerations from cutting with knives). After a few days of treatment and stabilization he was referred back to the inpatient psychotherapy treatment centre, and following a few weeks of suffering from the same range of symptoms, his condition improved dramatically. This improvement, which surprisingly stabilized over the next months, could not be explained by any other means by the staff of the inpatient treatment center. The patient was re-admitted to our clinic in November 2004 with similar symptoms but this time he told the psychiatric team he could now control himself much more before and during the upcoming flashbacks. Drug testing was negative with the exception of THC. When he was asked what his idea was about the improvement of his condition, he confessed that he had learned to smoke cannabis resin from some other inpatients. He had discovered that he could prevent dissociative states by smoking cannabis when he first felt reactivation and intensification of traumatic memories experienced as flashbacks. Although he still experienced flashback phenomena after the use of cannabis, he would smoke cannabis to alter their course and intensity. The patient described that cannabis use would assist him with the increased ability to maintain cognitive control. Though it did not eliminate traumatic images, cannabis allowed the patient to view them on an 'inner screen' from a distance. It should be mentioned that this patient never underwent specific PTSD-screening procedures for the treatment of intrusive flashback memories.[21] The urge for self-mutilation was also reduced when he smoked cannabis immediately after experiencing flashbacks. Sometimes he could not only prevent the urge to self-mutilate afterwards but could often feel cheerful instead. The patient stated that he found cannabis more useful than lorazepam because it worked better at targeting the very symptoms that were otherwise intensely painful and contributed to his self-injury and because he noticed that this occasional use of cannabis did not affect vigilance when compared to lorazepam. This was confirmed by his therapists and the fact that there had been no need for treatment of self-mutilation since he had started using cannabis in these critical states. His therapists at the inpatient psychotherapy treatment centre were not aware of his use of cannabis but noticed and charted the patient´s improved self-control and stability. It is evident from the case history that the patient experienced reduced stress, less involvement with flashbacks and a significant decrease of anxiety. In the following paragraphs, some key issues will be discussed that might be relevant for the mitigating effects of acute PTSD symptoms experienced by this patient. It is worth noting that the cannabis used was cannabis resin from turkey which is known to contain THC and a nearly equivalent amount of CBD.[22]

The pathophysiology of PTSD

PTSD is a serious disorder that is usually induced by one or more traumatic events. These events are typically characterized by their overwhelming character which have an impact on the organism

or psyche that renders the person unable to handle the impact at the moment of occurrence. A typical example is rape and a significant number of people who have experienced one or more such traumatogenic situations will develop symptoms of PTSD. These usually consist of a heightened level of general central nervous system (CNS) arousal, sleep disturbances, nightmares, psychological instability, depression, anxiety, avoidance behaviour, emotional numbing, and repeated intrusions of parts of the experience into consciousness ('flashbacks').[23]

On the neurophysiological level, patients with PTSD develop a hyperactivity of the amygdala[24] which is a central part of the fear network which is involved in the assessment of threat-related stimuli.[25,26] In these patients, the amygdala is especially hyper-responsive to the presentation of trauma-relevant stimuli.[27] Morphological studies were inconclusive in regards to structural changes in the amygdala,[28] but the amygdala appears to be implicated in extinction learning. The hippocampus is involved in learning and explicit (declarative) memory, working memory,[29] episodic memory,[30] and has also a role in the regulation of stress.[31] A decreased hippocampal volume of gray matter is a regular finding in chronic PTSD patients and there is evidence that elevated blood flow in the hippocampus is related to episodic, spatial and contextual memory and emotional responses. Activity in parahippocampal structures can be triggered by symptom provocation tests like trauma-relevant imagery.[32]

In contrast to over-activation of the amygdala, hippocampus and parahippocampal structures, and the anterior cingulate cortex (ACC), show decreased activity in acute[32] and chronic PTSD.[28,33] On the other hand, a sub-region of the ACC, the dorsal ACC (dACC), which is involved in emotional regulation, recall of emotional experiences and processing of emotional responses,[34] is over-activated in PTSD during symptom provocation tests (e.g. playing combat sounds to war-related traumatized PTSD patients).[35] In a study using [^{15}O]H_2O-PET, the exposure to traumatic imagery, which induces flashback-like memory, activated the medial posterior orbitofrontal cortex, the insular cortex, the anterior temporal pole, and the medial temporal cortex.[27,36] A deactivation of the rostral ACC was also observed.[37] The ACC is part of the medial prefrontal cortex and is also involved in the process of fear extinction conditioning.[38,39] The insular region mediates somatosensory processes, feelings and recall of emotional events such as emotional memory. A comparison between acute and chronic PTSD showed that acute PTSD displayed a more extended and unstable pattern of activation while chronic conditions included more circumscribed and stable neurofunctional abnormalities.[36] Flashback memories are typically induced by inner or outer stimuli which activate the amygdala and induce the retrieval of 'unmetabolized', but instead hypermnestically stored, memories from the hippocampus. Another important structure for the maintenance of PTSD is the ventromedial prefrontal cortex, which plays a major role in extinction learning ('forgetting') by interacting with the amygdala in a reciprocal fashion, i.e. leading to inverse correlation during emotional activity. This happens via inputs to inhibitory GABAergic cells that block information flow from the amygdala's lateral to central nucleus[40] and also regulates the hippocampus in regard to extinction recall.[41,42] The medial prefrontal cortex appears to be hypoactive in PTSD.[33,43]

From the evidence cited, the hypothesis was formed that a major cause for the persistent inappropriate fear responses and the diminished extinction of conditioned fear in PTSD patients may include under-activation of the ACC and the medial prefrontal cortex which may help to explain the emotional dysregulation observed in these patients. Learned fear associated with PTSD can persist for tens of years. Therapeutic interventions for PTSD include extinction learning and psychotherapeutic approaches for PTSD aim to strengthen the function of the medial prefrontal cortex to enhance the capability of extinction learning and to break the cycle of an over-activated fear system (amygdala, hippocampus, parahippocampal structures) while under-activating ACC and medial prefrontal cortex. Animal experiments have shown that extinction learning and recall involve different cellular mechanisms and possibly different brain regions.[44]

Possible mechanisms involved in the effects of cannabinoids in PTSD

The endocannabinoid system

The plant *Cannabis sativa* has been used by humans for thousands of years because of its psychoactive properties. The major psychoactive ingredient of cannabis is THC, which exerts effects in the brain by binding to a G-protein-coupled receptor known as the cannabinoid CB_1 receptor.[45] Two putative endocannabinoid ligands, arachidonylethanolamide (anandamide, AEA) and 2-arachidonylglycerol (2-AG), have been identified as major endogenous transmitters of the endocannabinoid system (eCB). The eCB system is distributed throughout the brain and regulates synaptic release of excitatory and inhibitory neurotransmitters. A key role of the eCB system is the activation of the CB_1 receptor which is widely represented in the brain showing a 10-fold higher distribution level in comparison with opioid receptor levels. Endocannabinoids such as AEA and 2-AG that interact with these receptors are post-synaptically synthesized signalling molecules and are not stored in vesicles. Instead, they appear to be generated on demand and liberated to act in a retrograde fashion on presynaptically localized CB_1 receptors.[46] Recent research revealed that the eCB system is homoeostatic in that it prevents extreme cortical excitation and inhibition and that it may be dysfunctional in some mental disorders. eCB signalling is widely distributed throughout corticolimbic circuits that are linked to the stress response. The general level of cortical excitability is determined by the neurotransmitter systems using GABA and glutamate. Stress, especially linked to some severe psychiatric disorders like PTSD, may produce an imbalance in the eCB system. This system serves as a modulator, comparable to a 'dimmer switch' that helps to prevent excessive excitatory or inhibitory activity.[18] Since the discovery of the endocannabinoid system a growing body of psychiatric research has emerged focusing on the role of this system involved in major psychiatric disorders like schizophrenia, bipolar disorder, major depression and anxiety disorders.[47] For example, the CB_1 receptor antagonist rimonabant was reported to cause depression and anxiety in a significant proportion of psychiatrically normal subjects.[48]

Cannabis and exocannabinoids

Three major exocannabinoids are THC, CBD and cannabinol (CBN) and represent the main constituents found in cannabis resin.[49] THC is the major psychoactive constituent and is responsible for the mood and consciousness-changing effects.[50] Reports concerning anxiolytic properties are inconsistent, and in some subjects, anxiogenic effects can be generated instead.[51,52] Besides THC, CBD is the main non-psychoactive phytocannabinoid found in *C. sativa* which can constitute up to 40% of its

extract. CBD has anxiolytic, anti-psychotic and anti-convulsant effects and antagonizes the intoxicant and psychotomimetic actions of THC and has opposite effects on regional cerebral blood flow (rCBF) when compared to THC.[53,54] Recent reviews indicate that CBD is a promising candidate for the treatment of some neuropsychiatric disorders.[55–57] CBD also facilitates extinction in a contextual aversive conditioning model following intracerebral ventricular administration.

Cannabis and anxiety

It has been argued that the neuronal circuitry underlying fear conditioning has similarities to that responsible for fear-related clinical conditions, such as PTSD.[25] Moreover, behavioural therapies for PTSD/anxiety, including systematic desensitization and therapies relying on imagery, also share features of fear extinction. Although high doses of intravenous THC may appear to increase anxiety in humans,[58] low doses attenuate anxiety-related responses in animal models.[59] It was also shown that anxiety disorders may make people more vulnerable to cannabis abuse and dependence.[60–62] This vulnerability may depend on an increased sensitivity towards anxiety and the probability that these individuals may cope with their aversive anxiety by using cannabis was found to be higher.[63] Cannabis dependence increases the risk for panic disorders, but the causal direction was not definitely disentangled.[64] It is discussed in how far a 'repeated affect-relevant learning with aversive interoceptive cues' through the use of cannabis may be a key risk mechanism for maintaining cannabis dependency and relapse after treatment.[64]

There appears to be a lack of clinical investigation regarding eCB-activity and anxiety but preclinical and clinical data strongly suggest that anxiety is associated with a decreased endocannabinoid tone leading to excessive cortical excitation, particularly in stressful situations. The influence on anxiety is thought to be mainly mediated by CB_1 receptors, but also possibly by CB_2 receptor and G-protein coupled receptor activation which appears to involve decreased anxiety in a variety of rodent assays such as the elevated plus maze test. The fact that these effects are partially inconsistent may depend on a number of factors including regional endogenous tone, type of test, and dosage.[65] Mice, when exposed to a stressful environment, display a stronger anxiety response than their CB_1 knockout counterparts.[66,67] In addition, anxiolytic actions of benzodiazepines were observed to be absent in CB_1 receptor-knockout mice which presented increased anxiety-like behaviours. Thus, it was concluded that the CB_1 receptor played a pivotal role in the anxiolytic action of benzodiazepines.[68,69] Anxiolytic effects of CBD have also been demonstrated after microinjection into the dorsolateral periaqueductal gray, bed nucleus of the stria terminalis and prelimbic medial prefrontal cortex.[70–72] Additionaly, the CB_1 receptor antagonist rimonabant causes depression and anxiety in a significant proportion of psychiatrically healthy normal subjects.[48] Clinical studies have shown that CBD displayed anxiolytic properties, for example in subjects who showed anxiety of speaking in public.[73] In agreement with these findings, neuroimaging studies showed that CBD facilitated a change in brain activity in regions related to emotional responses. It impairs connectivity between the prefrontal and subcortical regions and attenuates responses to fearful faces in the maygdala and cingulate cortex[74] and furthermore decreases activation in the left amygdala-hippocampal complex and left posterior cingulate gyrus.[75]

Effects on hippocampus and memory

Endocannabinoids exert an amnesic effect and may be crucial for the extinction of aversive memories,[76,77] while blockage or knockout of the CB_1 receptor induce deficits of the extinction processes and supersensitivity to stress[78] by decreasing GABAergic function.[67] The mechanisms by which cannabinoids alter perception and memory have not been exactly elucidated. *In-vivo* recordings of populations and single neurons have shown that THC disrupts the synchrony of action potentials between hippocampal neurons with only marginal effects on average firing rates.[79] The hippocampal formation has an unusually high density of CB_1 receptors[80] and these are involved in both glutamatergic and GABAergic presynaptic processes.[81] CB_1 receptors are present on certain peri-terminal axons at astonishingly high densities,[82] enabling endogenous and exogenous cannabinoids to potently inhibit action potential-evoked GABA and glutamate release by means of CB_1 receptor-mediated inhibition of N-type presynaptic calcium channels. Thus, cannabinoids can dramatically depress fast synaptic communication in the hippocampal network leading to a functional decoupling of neurons. Robbe and co-workers[79] found that administering THC depressed hippocampal and neocortical electroencephalograms in rats at multiple frequencies. The effects of cannabinoids on gamma oscillations are especially important, because neurons form *ad hoc* assemblies defined by synchronous action potential firing.[83] These assemblies are thought to be tasked with the representation, storage and retrieval of information and memories. Hippocampal theta- and gamma oscillations are thought to be critical in working memory, the encoding of episodic memory, and in the coordination of neuronal discharges across regions.[84] Exogenous cannabinoids disrupt the induction of hippocampal long-term potentiation (LTP) and impede on behavioural learning, potentially including strength of association between stimuli and fear or anxiety. By reducing synchronous firing, exogenous cannabinoids may reduce the associational activation of synapses that induces LTP.[85] It has to be discussed in how far cannabinoids mediate disorganization of synchronized cell assemblies, and by doing so, leading to decreased hippocampus-dependent memory performance. A major implication of these data is that the synchrony of spike timing in neuronal assemblies is a necessary component of proper hippocampal function and that THC may reduce anxiety by reducing activation of hippocampal networks that retrieve fear-related memories, as when triggered by associated stimuli.

Involvement of endocannabinoids in fear extinction

A large body of work has established that a small region of the brain, i.e. the amygdala, is crucial in acquiring and, possibly, storing the memory of conditioned fear.[26] Endocannabinoids exert an amnesic effect and are crucial for the extinction (forgetting) of aversive memories[76,77] while blockage of the CB_1 receptor induces deficits on the extinction of aversive memories and supersensitivity to stress.[78] Extinction or reduction of fear responses (i.e. required by trauma) may be generated on a neurobiological level through synaptic plasticity mediated by NMDA receptors[86] but other mechanisms of extinction may also be involved.[87] Marsicano and colleagues[76] proposed a mechanism of extinction involving the eCB and CB_1 receptors which are some of the most abundant neuromodulatory receptors in the CNS and are expressed at high levels in the limbic system, cerebellum and basal ganglia.[88,89] The main psychopharmacological effects of exogenous cannabinoids

(sedation and changes in memory) have been correlated with the presence of CB_1 receptors in the limbic system and striatum.

Endocannabinoids also play a role in inhibiting neurotransmitter release. The research carried out by Marciano *et al.*[76] demonstrated the impact of endocannabinoids on learning and plasticity. It was shown that CB_1 receptor knock-out mice could learn and later recall association of a tone with a foot shock but were unable to extinguish the memory, i.e. their emotional response to the tone. These authors found that during the extinction period, the levels of endogenous AEA and 2-AG were raised in the basolateral amygdala in mutant and normal mice which implied a role for endocannabinoids in the extinction of conditioned fear. CBD also facilitated extinction in a contextual aversive conditioning model after intracerebral ventricular administration.[54]

Effects of endo- and exocannabinoids via stress-related hormonal systems

Stress can be defined as confrontation with stimuli that presents a challenge to homoeostasis, typically a perceived stress to the well-being of the organism. In humans, acute and chronic stressful situations correspond with the secretion of glucocorticoid hormones. The paraventricular nucleus (PVN) of the hypothalamus releases corticotropin-releasing hormone (CRH) and the anterior pituary gland releases the adrenocorticotropic hormone (ACTH) into general circulation. Subsequently, glucocorticoid hormones, such as cortisol (from the adrenal cortex), are released to mobilize energy stores and to induce a range of effects on cardiovascular, immune, metabolic, and neural systems that facilitate optimal responses to aversive stimuli.[90] Although this may have adaptive functions in the short term, in cases of repeated stress exposure, prolonged glucocorticoid secretion can produce deleterious effects on metabolic, immune, cardiovascular and neurobiological functions.

Both hippocampus and PFC exert inhibiting effects on the hypothalamic-pituitary-adrenal (HPA) axis whereas antidepressive agents can normalize its hyperactivity. Furthermore, it has been shown that eCB signalling responds to and regulates the activity of the HPA axis which governs their secretion of stress hormones.[91] The eCB system, maintaining homoeostais of the stress system, can activate as well as terminate the HPA axis response to acute and repeated stress. Accumulating evidence indicates that the eCB tone provides a steady-state inhibition of the HPA axis activity.[92] Prominent in these behavioural stress responses is the interaction between eCBs and the HPA-axis. Data indicate that glucocorticoids induce eCB signalling through a rapid non-genomic process in CRH neurons of the PVN.[93] This induction of eCB signalling inhibits glutamatergic inputs to CRH neurons and thus decreases the excitatory drive to the HPA axis.[92] Glucocorticoids are self-regulated through negative feedback and eCB mediates glucocorticoid fast feedback mechanisms. Fast feedback inhibition of HPA axis stress responses by direct glucocorticoid action at the PVN of the hypothalamic rapidly inhibits restraint-induced ACTH and corticosterone release consistent with feedback actions at the cell membrane.[94] It was demonstrated that following repeated exposure to stress AEA is persistently decreased throughout the corticolimbic stress circuit whereas 2-AG is elevated (exclusively in the amygdala) in a stress-dependent manner.[95] This divergent regulation of AEA and 2-AG contributes to distinct forms of HPA axis habituation. Inhibition of AEA hydrolysis or intra-amygdala administration of a CB_1 receptor antagonist before the final stress exposure prevented the repeated stress-induced development of basal hypersecretion of corticosterone.[91] Thus, there is evidence for both GABAergic and CRH-mediated mechanisms involved in the anxiolytic effects of THC.

Reduction of anxiety and amygdala reactivity and the eCB system

The amygdala has been identified as one of the primary limbic structures involved in activating the HPA axis in response to stressful stimuli. There is also accumulating evidence that glucocorticoid-mediated induction of eCB signalling is also a relevant feature, because glucocorticoids enhance the long-term consolidation of emotionally arousing experiences.[92] Presence of eCB signalling within stress-sensitive nuclei of the hypothalamus as well as upstream limbic structures, such as the amygdala, suggests a role in regulating the stress response. Administration of CB_1 antagonists into the basolateral nucleus of the amygdala (BLA) blocks the ability of corticosterone to facilitate aversive memory consolidation[96] which highlights an important role of the eCB system in this complex adaptive process. During extinction training, but not initial fear conditioning, eCB levels in the amygdala, but not in the prefrontal cortex, were elevated. Mice lacking the CB_1 receptor exhibit prolonged expressions of fear behaviours during extinction training.[76] In mice exposed to brief inescapable electric foot shock subsequently presented a neutral tone, the CB1 receptor-deficient mice failed to suppress the conditioned fear response when the shock was stopped and showed persistent fear on on repeated tone exposures.[97] From these studies it was concluded, that 'the feardampening effects of eCBs become evident only in highly aversive situations and are independent of CRH and corticosterone action'.[97] The dampening of anxiety and over-arousal, especially in regard to inducing flashback memory appears significantly reduced in the case described in the beginning of this review.

During the adaption to stress and aversive stimuli the amygdala shows no change in 2-AG in response to acute stress.[98–100] However, following repeated stress/aversive stimuli a 2-AG increase was progressively observed[99] followed by decrease after 1 h and complete reversal within 24 h of exposure.[101] As far as habituation to homotypical stress is concerned, this reaction pattern is critically involved in the habituation of the HPA axis. Accordingly, the increase of 2-AG correlates directly with HPA axis suppression and the local administration of a CB_1 receptor antagonist into the BLA reversed the expression of stress habituation.[101] Transient augmentation of 2-AG signalling upon repeated stressor exposure dampens excitatory inputs to the BLA by decreasing outflow of the amygdala, which would include stimulation of the HPA axis.[101] This would be consistent with corticosterone inhibition of glutamatergic inputs to the BLA through an eCB-mediated mechanism but only in animals with history of previous stress exposure.[102] BLA administration of CB_1 antagonists blocks the ability of systemically administered corticosterone to facilitate aversive memory consolidation.[96] Glucocorticoids recruit eCB signalling in the BLA to modulate aversive memory consolidation. The amygdala's GABAergic system is known to modulate memory storage[103] and activation of CB_1 receptors decreases GABA release via rapid inhibition of Ca^{2+} entry into the terminals.[104] A recent fMRI neuro-imaging study in humans demonstrated that THC discretely attenuated localized limbic (amygdala) reactivity to threatening stimuli without affecting performance on other complex tasks.[105] Interestingly, these results resemble those shown with lorazepam.[106]

Cannabinoids decrease CRH levels in the central nucleus of the amygdala and decreased CRH levels are associated with decreased aversive stress responses in animals[107] and humans.[108] It was also demonstrated by Marciano *et al.*[76] that basolateral amygdala neurons present in normal mouse brains are capable of releasing GABA when stimulated under low-frequency conditions. This can lead to a long-term reduction in the release of GABA which in turn leads to less inhibition of the connecting pyramidal neurons. This long-term depression (LTD), a type of synaptic plasticity, was completely blocked by the CB_1 receptor antagonist rimonabant (SR141617) and absent in CB_1-deficient mice.[109] This finding implies a reduction of GABA release in the basolateral amygdala, thereby helping to extinguish the fear-conditioned response or reduction of anxiety.[58] CBD has been shown to attenuate neurophysiological responses to fearful faces in the amygdala as shown by fMRI[74] and, in addition, reduced activation in the left amygdala-hippocampal complex and left posterior cingulate cortex.[75]

Antidepressant effects of cannabinoids

Another significant set of symptoms observed in PTSD patients include depressive mood and stressful sleep disorders. Several lines of evidence suggest that cannabis may have antidepressant effects. Nevertheless, no clinical trials have been published to date on the use of cannabinoids for the treatment of affective disorders although anecdotal reports have described antidepressant properties of cannabis.[8] Some methodological limitations present in the very few human studies currently available make interpretation difficult. Antidepressive agents increase monoamine neurotransmitters such as serotonin and noradrenaline[110] and normalize the hyperactivity of the HPA axis, which also involves the eCB system. In a study designed to look for possible uses of cannabis resin as an antidepressant in recreational users[111] it was concluded that patients may be found who use marijuana for self-treatment of depressive symptoms. Degenhardt *et al.* argued that there was little evidence for an increased risk of later cannabis use among people with depression, and hence little support for the self-medication hypothesis.[112]

Several authors have reported altered endocannabinoid levels involved in the precipitation of depression.[113] The pharmacological enhancement of endocannabinoid activity at the CB_1 receptor level appears to exert an antidepressant-like effect in some animal models of depression. CB_1 agonists significantly increase the firing activity of neurons in the dorsal raphe, thus enhancing serotonin neurotransmission.[114,115] Stimulation of CB_1 activity was shown to increase firing in the locus coeruleus as well as the release of norepinephrine (NE) in the prefrontal cortex (PFC)[115,116] which implies antidepressant activity. Additional evidence comes from co-treatment with α- and β-adrenergic receptor antagonists that were found to attenuate antidepressive effects induced by chronic administration with CB_1 receptor agonist.[117] Moreover, it appears that CB_1 receptors modulate the effect of the selective serotonin reuptake inhibitor citalopram on extracellular serotonin levels in the rat prefrontal cortex.[118]

Sleep-enhancing effects of cannabinoids

It is well known that PTSD includes a pathologically hyperarousal syndrome which leads to serious sleep problems, especially with sleep onset and increased numbers of awakenings during the night. *C. sativa* has been utilized for the treatment of pain and sleep disorders since ancient times. Early studies reported an earlier sleep onset, decreased REM sleep and an increase in stage 4 sleep with the ingestion of THC in healthy subjects. Both drugs reduced eye movement density with some tolerance developing to this effect.[119–121] Marijuana effects on sleep were virtually identical to those produced by the same doses of THC.[120,121] However, an issue that requires further clarification in this context is that it was unknown whether these preparations contained CBD. Controlled studies have demonstrated that with orally administered dosage levels of 10, 20 or 30 mg THC the time needed for mild insomniacs to find sleep was minimized. Twenty mg THC were most effective and reduced the time of falling asleep by 62 min (placebo: 180 min *vs* 118 min). Higher doses did not improve this any further.

A more recent study which examined the relationship of THC (15 mg p.o.) and a combination of THC and CBD (5 mg or 15 mg p.o. of each substance) on sleep in a cross-over design found a very differentiated impact on sleep patterns. According to this study, no significant effects of pure THC on the sleep measures were observed, but a decrease of rapid eye movement (REM) sleep periods and REM duration as well as a decrease of stage 3 sleep was found instead. On the other hand, the combination of THC and CBD led to highly significant decreases of REM sleep (placebo: 84.75 min; THC 15 mg and CBD 15 mg: 61.88 min) at higher dosage levels, but at the same time, an increase of duration of wakefulness (placebo: 17.06 min; THC 15 mg and CBD 15 mg: 41.06 min) was also observed. No difference in latency of sleep onset and number of awakenings with any of the substances or combinations was found compared to placebo.[122] The authors noted the occurrence of sleepiness on the next morning following THC (15 mg) administration but also when the higher combined dose was given. When evaluating the psychomotor and memory performance on the next day clinically significant effects of the drugs were not detected. Surprisingly, in rats the endocannabinoid anandamide did not block the effects of CBD.[123,124] The mechanism of action of CBD on sleep modulation remains to be elicited[125] but it was speculated that CBD may modulate wakefulness by via an activation of neurons in the hypothalamus and the dorsal raphe nucleus.[124] Anandamide was observed to decrease wakefulness in addition to increases in slow wave sleep and REM sleep in rats.[124] When the action of anandamide was blocked by the CB_1 receptor antagonist SR 141716A, i.e. 15 min prior to anandamide administration, these anandamide-induced changes in sleep were not observed, hence providing indication that the CB_1 receptor was a major target for the sleep-inducing actions of anandamide.[125]

According to the study of Cousens and DiMascio[126] there was a decrease in the number of sleep interruptions, especially in the first third of the night which suggested that the hypnotic actions of THC were relatively short-lived. Some subjects complained in the morning about a mild to moderate feeling of being hungover or being stoned, but subjects who received the 20 mg dose did not observe any interference with their daily work function.[126] More recent studies showed THC and cannabis to be effective in sleep disorders and that they were well tolerated[127] and a low nabilone (THC) dose given once per day at bedtime was suggested as a possible alternative to amitriptyline.[128] No tolerance on pain or sleep, nor a need for dosage increases have been observed.[3] When intracerebroventricular administrations of CBD (10 μg/5 μL) were employed in rats during the lights-on sleeping period, an increased wakefulness and a decreased REM sleep was observed although sleep changes during the dark phase were not observed.[124] The decrease of REM sleep in humans may contribute to the fact that most individuals report a less frequent occurrence of dreams,

especially nightmares. The main conclusion from experiments carried out in humans with cannabis resin/marijuana, which includes a mix of cannabinoids (usually mainly THC and CBD), is that increases in sleep appear to be consistent features.[119,129–131]

An unexplained fact is the significant increase of 'strange dreams' for more than two weeks during withdrawal from heavy cannabis smoking,[132] which does not appear to depend on REM rebound. In a study examining regular cannabis users during a few days abstinence period, participants had a mild to moderate degree of decreased sleep efficiency, total sleep time, percent time spent in Stage 1 and Stage 2 sleep, REM latency and subjective sleep quality, as well as increased sleep latency and time spent in REM sleep when compared to these patterns when using cannabis.[133] In rats, which were sleep deprived for 24 h, it was demonstrated that the usually seen REM rebound was very much reduced when the CB_1 receptor antagonist SR 141716A was given before sleep.[134] However, a reduction in REM sleep observed with cannabis does not seem to be a consistent finding,[135,136] which may point towards different possible implications. It may lead to decreased periods of wakefulness and nightmares, although less REM sleep is also discussed to alter affect regulation and memory-related processes[137–139] and that it may also play a role in depression.[140]

Discussion

It seems obvious from more recent studies of clinical and non-clinical populations that cannabis is used by a significant number of PTSD patients in the attempt to cope with their symptoms.[10–15] It appears that through different levels of actions (physiological, transmitter and molecular) eCBs are involved in the etiological mechanisms of certain mental disorders. The field of research investigating the eCB system is growing rapidly. The effects of cannabinoids, even if in some important aspects not well researched in humans, are complex and include effects on mood, stress and distress mechanisms, mainly involving the HPA axis and its regulation via fast feedback/presynaptic mechanisms. Endocannabinoid systems also show direct effects on major limbic and paralimbic structures, especially in fear conditioning, habituation and extinction. Therefore, it appears that modulation of the eCB system might be a rewarding target for psychopharmacological drug development. It might even be possible that some cannabinoids may offer potential to compete with commonly employed antidepressive agents, at least in some respects (Table 1).

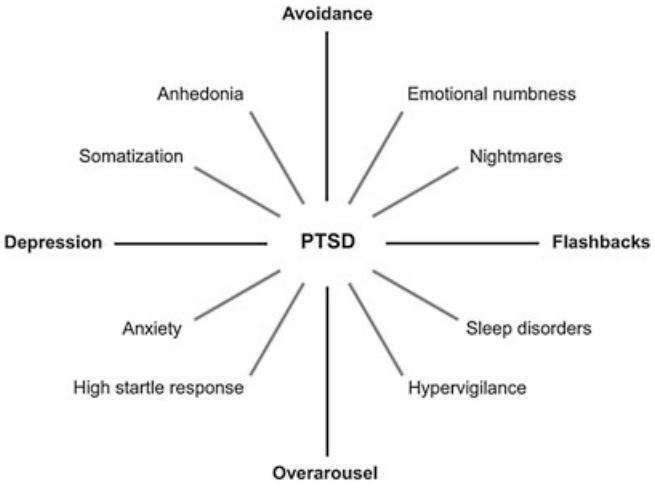

Figure 1. Symptom clusters typically involved in PTSD.

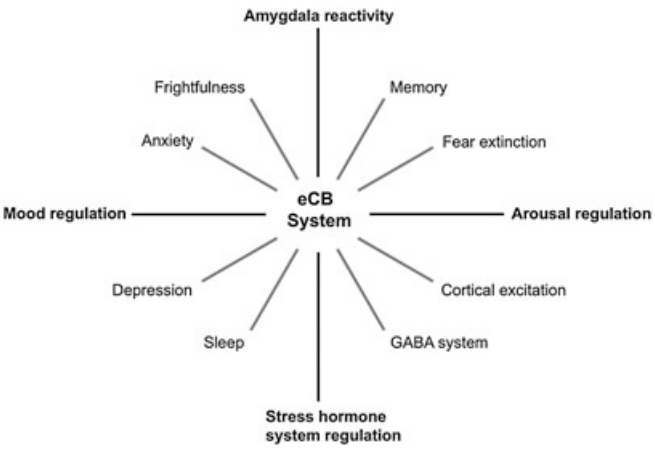

Figure 2. Involvement of the endocannabinoid system in neurobiological systems.

If one looks at the symptoms specific for PTSD (Figure 1) it also appears that effects at multiple levels that involve eCB signalling may be helpful when coping with symptoms of PTSD (Figure 2). Reduction of over-arousal, nightmares, sleep disorder, flashbacks as well as antidepressant and anxiolytic effects, may be achieved.

Table 1. Effects of Cannabis (THC, CBD) and antidepressants on symptoms of PTSD based on data given in cited references and clinical experience. + = effective; ++ = very effective

Symptom	Cannabis resin (THC + CBD)	Antidepressants (SSRI-type)	Antidepressants (Trimipramine/Amitriptyline-type)
Overarousal	+		+
Flashbacks	++ (frequency and intensity)	+	
Nightmares	++ (less REM)		+ (mirtazapine)
Anxiety	+	+	
Depression	+	++	++
Sleep disorders			
Sleep onset	++		++ (mirtazapine, amitriptyline)
Awakenings during night	++		+

Multiple effects associated with cannabis resin appear to act synergistically to reduce some symptoms of PTSD and might offer potentials for new psychopharmacological treatments. Therefore, PTSD subjects may opt to self-medicate by using cannabis.

In the case report presented in this review, the patient displayed a grave pathology involving anxiety, dissociation and heavy flashbacks as a consequence of PTSD. When he began to use cannabis he observed that he could handle his symptoms much better and that he was able to refrain from getting too involved with the flashbacks. The patient described this as being able to look at them from a distance, i.e. 'from outside'. His anxiety was also much more manageable, and as a consequence, he was able to handle the situation much better while exercising greater control. In the case where he was able to detect an upcoming flashback early enough he was able to stop the flashback from appearing by smoking cannabis resin. One possible explanation might include a reduced involvement of the amygdala (and hippocampus) in an overreaction that would otherwise produce panic and an overwhelming altered state of dissociation, including intrusive flashback memory. The patient found himself in control and was able to reduce his suffering.

It should be noted that although cannabis has been used as a psychopharmacological agent for centuries deleterious effects are commonly observed in some individuals, including dependence and worsening of life conditions associated with regular cannabis use. Even if excluded from the DSM-IV-TR, there is growing evidence that a significant cannabis withdrawal syndrome (mild to moderate symptoms of sleep difficulty, strange dreams, irritability, restlessness) may appear after longer time of daily smoking of cannabis in 60-75% of the users, but its clinical significance is still debated.[132]

It has been hypothesized that PTSD is maintained by amygdala hyperreactivity[141] and that cannabis may dampen the strength or emotional impact of traumatic memories through synergistic mechanisms that might make it easier for people with PTSD to rest or sleep and to feel less anxious and less involved with flashback memories. The presence of endocannabinoid signalling systems within stress-sensitive nuclei of the hypothalamus, as well as upstream limbic structures (amygdala), point to the significance of this system for the regulation of neuroendocrine and behavioural responses to stress. The eCB system is involved in activation and termination of the HPA axis reactions to acute and chronic stress.

Conclusions

This review provides an overview of accumulating clinical and preclinical evidence that cannabinoids may mitigate some major symptoms associated with PTSD. A case study was presented of a patient with severe PTSD symptoms, who learned to smoke cannabis resin in order to cope with grave PTSD symptoms and who benefitted enormously from doing so. The accumulating evidence points towards diverse actions where the endocannabinoid system is involved in different neurobiological systems critical for the complex pathogenesis of PTSD. Findings from studies suggest that by altering fear conditioning, memory systems, general CNS arousal, mood, and sleep, exogenous cannabinoids may hold potential for the treatment of people with PTSD.[17] While it seems clear that that consumption of cannabis products may not be well tolerated in all individuals, more research is needed to reach definite conclusions about a therapeutic potential of cannabinoids in PTSD.

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SHORT COMMUNICATION

CNS Neuroscience & Therapeutics

The Use of a Synthetic Cannabinoid in the Management of Treatment-Resistant Nightmares in Posttraumatic Stress Disorder (PTSD)

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Keywords
Cannabinoids; endocannabinoids; nabilone; nightmares; PTSD.

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This is the report of an open label clinical trial to evaluate the effects of nabilone, an endocannabinoid receptor agonist, on treatment-resistant nightmares in patients diagnosed with posttraumatic stress disorder (PTSD). Methods: Charts of 47 patients diagnosed with PTSD and having continuing nightmares in spite of conventional antidepressants and hypnotics were reviewed after adjunctive treatment with nabilone was initiated. These patients had been referred to a psychiatric specialist outpatient clinic between 2004 and 2006. The majority of patients (72%) receiving nabilone experienced either cessation of nightmares or a significant reduction in nightmare intensity. Subjective improvement in sleep time, the quality of sleep, and the reduction of daytime flashbacks and nightsweats were also noted by some patients. The results of this study indicate the potential benefits of nabilone, a synthetic cannabinoid, in patients with PTSD experiencing poor control of nightmares with standard pharmacotherapy. This is the first report of the use of nabilone (Cesamet; Valeant Canada, Ltd., Montreal, Canada) for the management of treatment-resistant nightmares in PTSD.

Background

The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR), defines posttraumatic stress disorder (PTSD) as the development of characteristic symptoms following exposure to an extreme traumatic stressor, involving direct personal experience of an event that involves actual or threatened death or serious injury or other threat to the physical integrity of another person, or learning about unexpected or violent death, serious harm or threat of death, or injury experienced by a family member or other close associate. The person's response must involve intense fear, helplessness, or horror (in children, disorganized or agitated behavior). There are many characteristic symptoms of PTSD including the persistent, intrusive recollections or re-experience of the original event (via dreams or nightmares and dissociative flashbacks), numbing and avoidance, and increased arousal [1]. The experience of these symptoms leads to functional impairment.

Although PTSD is often associated with military casualties, the majority of cases are related to traumatic events occurring in the general population. Such events may include physical or sexual abuse, traffic or natural disasters, and interpersonal violence. The lifetime prevalence of PTSD is 8.2% in the United States, and a Canadian study puts this rate at 9.2% [2,3]. PTSD's lifetime prevalence is higher than that of other anxiety disorders, including panic disorder, obsessive compulsive disorder, and generalized anxiety disorder.

Guidelines for the management of PTSD now exist [4]. However, recommended first-line and second-line agents, used alone or in combination to treat symptoms including nightmares, often show limited effectiveness in many patients. Subsequently, some patients may continue to experience symptoms, including debilitating nightmares, for years or decades. The negative impact of nightmares and the side effects of some of the current psychotherapeutic medications may potentiate other symptoms of PTSD, including those related to anxiety

and depression. Other comorbid psychiatric conditions may also worsen. Commonly, patients with PTSD are receiving more than one medication. Polypharmacy is associated with the potential for side effects and drug interactions, thus possibly creating compliance and quality-of-life issues. On the basis of these experiences, there is a definite clinical need for a medication that is effective in treating nightmares related to PTSD, with positive effects on sleep and little potential for side effects or drug interaction.

Selective serotonin reuptake inhibitors (SSRIs) are considered first-line agents in the pharmacological treatment of PTSD in the United States (e.g., paroxetine and sertraline). Second-line agents include venlefaxine, prazosin, monoamine oxidase inhibitors, and tricyclic antidepressants. Other agents used in PTSD include atypical antipsychotics and anticonvulsants [5].

Sleep disturbances, mainly insomnia and nightmares, are present in about 70% of those with PTSD. The estimates of nightmares vary from 24.8% [6] to 60.0% [7].

Various medications have been used in attempts to control PTSD sleep disturbances, including nightmares. A review of the abovementioned classes of medications, as well as other specific agents such as clonidine and cyproheptadine, concludes, "to date an insufficient number of controlled studies are published to formulate evidence-based guidelines. Drawing on the available data it can be concluded that there is limited but promising evidence for prazosin and olanzapine for managing PTSD nightmares and insomnia" [8]. That article also points out that objective parameters for insomnia and nightmares need to be developed. The fact that so many agents have been used in attempts to manage nightmares highlights that management of these is difficult, and that there is room to explore other potentially useful classes of medications. Anecdotal reports of relief from psychiatric symptoms, with the use of marijuana or a pharmaceutical endocannabinoid receptor agonist, have created interest in investigating the role of the endocannabinoid system in PTSD and other mood disorders [5]. The endocannabinoid system has been implicated in the control of various behaviors including eating, addiction, and memory and in mediating both anxiolytic effects and pain responses [6–8]. Endocannabinoids are thought to exert an effect through a variety of interactions with the CNS related to PTSD. These include the hypothalamic–pituitary–adrenocortical (HPA) axis, function of the hippocampus and amygdala, and control of cortical regulation of memory processes [9–11].

The endocannabinoid system comprises two G-protein-coupled receptors (CB_1 and CB_2), possibly one or more atypical receptors, and several ligands (notably anandamide and 2-arachidonolglycerol [2-A]). The CB_1 receptor is distributed primarily within the CNS, particularly in the cerebellum, basal ganglia, amygdala, cerebral cortex, and hippocampus [12,13]. The CB_2 is mostly distributed peripherally [13,14]. The cannabinoid receptors show pronounced selectivity in their binding and even have distinct binding sites for different classes of ligands [14]. This selectivity may partially explain why different agonists for the same CB receptor show differing therapeutic and side effect profiles. For example, at therapeutic doses, nabilone does not appear to produce the psychological high of inhaled marijuana.

Nabilone (Cesamet; Valeant Canada, Ltd., Montreal, Canada), an endocannabinoid receptor (CB_1 and CB_2) agonist, has been in use in Europe and Canada for over 25 years and was recently granted approval in the United States for the treatment of chemotherapy-induced nausea and vomiting. The identification and cloning of cannabinoid receptors in humans have led to a better understanding of the possible mechanisms of action of nabilone and support its potential use and safety in multiple clinical settings and various patient populations [12–26].

Rational for Therapeutic Trial of Nabilone in Patients with PTSD

Patients with PTSD can be desperate to obtain relief from their symptoms and frequently turn to self-medication, including the use of alcohol and cannabis. On the basis of observations published in a single case study that mentioned nabilone's reduction of nightmares when it was employed to replace a patient's use of smoked marijuana for the relief of PTSD symptoms [22], the author of this current report decided to initiate nabilone as pharmacotherapy for several patients whose nightmares were not adequately controlled with standard therapies. When the initial three patients experienced abolition of their nightmares, it was decided to use nabilone in subsequent clinical cases with similar presentations and record the effect on nightmares.

Methods

All 47 patients who agreed to participate in this clinical study had been referred to the author's private clinic for the management of PTSD by other physicians. The clinic specialized in the management of psychological trauma. Diagnoses for the study were confirmed by DSM-IV-TR criteria using a recognized PTSD questionnaire, the Posttraumatic Stress Diagnostic Scale [9]. All patients had at least a 2-year history of PTSD-related nightmares that had not responded to conventional therapies (Tables 1 and 2). Eligibility for this study stipulated that

Table 1 Population profile

	Total	%
Total number of patients studied	47	
Mean age, years ± SD	44 ± 9	
Range	26–68	
Women/men	27/20	57/43
Time since PTSD onset (range in years)	2–30	

Table 2 Type of trauma

	Total	%
Repetitive childhood trauma (sexual/physical abuse)	18	38
Civilian adult trauma (accident, rape, injury, workplace trauma, and life-threatening illness)	18	38
Combat-associated trauma	11	23
Total	47	100

current nightmare frequency was a minimum of once weekly.

Nightmares were considered "treatment-resistant" when these persisted in spite of conventional medications employed for PTSD. Although these medications provided relief for various PTSD symptom clusters, as reported by the patients in this study, nightmares persisted unchanged and continued to cause clinical distress.

The author had to rely on subjective reports of nightmare presence and subsequent relief with the use of nabilone since, at present, there is no reliable test to objectively measure the presence or intensity of nightmares.

All patients were informed that nabilone was a synthetic cannabinoid and approved only for antiemetic use. The patients were screened for previous negative experiences with marijuana use and were advised to not use marijuana while taking nabilone. Conditions that were contraindicated with the use of nabilone were excluded from the study (e.g., sensitivity to cannabinoids and psychotic reactions). All patients were on psychotropic medications for PTSD at the start of the study, and a decision was made not to discontinue any of these in order to study the effect of the addition of nabilone. The patients were carefully monitored for any adverse reactions. Potential benefits and side effects were discussed, and the patients were advised to discontinue nabilone if they experienced any uncomfortable side effects. Verbal consent was voluntary, and continuing psychiatric treatment was not contingent on being a volunteer.

Prior to starting nabilone, the patients were given a tracking sheet that asked them to record the intensity of nightmares from 1 to 5 (5 being the most intense) and hours of sleep and provided a space for comments about that night's sleep. This nightly charting began 1 week prior to commencing the trial and weekly thereafter until satisfactory results or the trial being ended due to side effects. Previous medications, which ranged from a single SSRI to polypharmacy, were not changed during the study.

The patients were started at a dose of 0.5 mg 1 h prior to bedtime (the first patient was started at 1.0 mg based on dose availability. Soon after, the 0.5-mg capsule became available). The patients were seen within 7 days of initiating nabilone in order to determine dose response and monitor for side effects. Titration of nabilone was indicated if the medication was well tolerated and effective control of nightmare symptoms had not been achieved. The patients continued to be seen weekly until a satisfactory response was achieved or nabilone was stopped due to side effects. All doses were kept below the maximum 6 mg daily, as per the Cesamet (nabilone) product monograph [28]. Patients having a positive response to nightmare cessation or reduction were permitted to continue nabilone therapy and were individually monitored for its use in ongoing therapy. All patients gave consent for a review of their clinical charts in order that their response to nabilone therapy be documented.

Results

For 47 patients, standard PTSD medications being maintained, the usual starting dose was 0.5 mg and was titrated up or down to effect. The average effective dose of nabilone was 0.5 mg one hour before bedtime, with an effective dose range of 0.2 mg to 4.0 mg nightly. Thirty-four (72%) patients experienced total cessation or lessening of severity of nightmares (28 patients had total cessation of nightmares and 6 had satisfactory reduction). The discontinuation of medication was successful in four patients following 4–12 months of nabilone therapy (nightmares did not return or returned at a reduced level, not needing further medication control), whereas the other patients experienced a recurrence of nightmares upon nabilone withdrawal (usually within the first two nights). These patients experienced control of nightmares once nabilone treatment was reinitiated. These patients were asked to attempt withdrawal at least every 6 months, but the therapy was ongoing at the time of this chart review. Three patients, who initially responded positively, were lost to follow-up.

In some cases, the benefits including an improvement in sleep time and a reduction of daytime flashbacks were subjectively noted. Several patients also stated that they no longer experienced nightsweats while on nabilone. Once effective relief of nightmares was achieved, no

further increase in nabilone was necessary (patients' doses remained stable). Thirteen (28%) patients experienced mild-to-moderate side effects (shortly following nabilone initiation), leading to discontinuation of nabilone therapy. The side effects experienced included lightheadedness, forgetfulness, dizziness, and headache.

Conclusion

A chart review of patients diagnosed with PTSD who were referred to a private psychiatric clinic suggests that the synthetic cannabinoid, nabilone, has beneficial effects beyond its official indication in regard to abolishing or greatly reducing nightmares that persisted in spite of treatment with conventional PTSD medications.

The subjects concomitantly received nabilone in addition to the one or more psychiatric medications that they were already taking for 2 years or more. No tolerance to nabilone was observed among the patients. This may indicate its potential longer-term safety and efficacy.

The author recognizes the limits of this study (e.g., there was no placebo control, the measurements were limited to subjective reports to nightmare changes, the study was on a small number of patients, and there was a selective bias by nature of referrals to a specific clinic from which the patients were selected). Nonetheless, on the basis of these retrospective findings, nabilone appears to be a significant treatment for nightmares in the PTSD population. This initial positive clinical report on 34 of the 47 patients will hopefully inspire other physicians to consider using nabilone in those with persistent PTSD nightmares. Nabilone should be evaluated further through randomized clinical trials involving PTSD patients, including studies looking at its effects on the full spectrum of PTSD symptoms. Baseline and follow-up polysomnography recordings for patients on nabilone therapy would likely provide useful information. In addition, nabilone's effect in other anxiety disorders and primary parasomnias may be the areas to investigate.

Addendum

Since this study was done, Health Canada has approved a 0.25-mg capsule of nabilone. This would be the preferred starting dose of this author. The United States has only the 1-mg capsule available, so dilution by a pharmacist for the initial doses is recommended. Available strengths may vary in different countries where nabilone is available.

Conflict of Interest

The authors declare no conflict of interest.

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The Endocannabinoid System as an Emerging Target of Pharmacotherapy

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Abstract*——The recent identification of cannabinoid receptors and their endogenous lipid ligands has triggered an exponential growth of studies exploring the endocannabinoid system and its regulatory functions in health and disease. Such studies have been greatly facilitated by the introduction of selective cannabinoid receptor antagonists and inhibitors of endocannabinoid metabolism and transport, as well as mice deficient in cannabinoid receptors or the endocannabinoid-degrading enzyme fatty acid amidohydrolase. In the past decade, the endocannabinoid system has been implicated in a growing number of physiological functions, both in the central and peripheral nervous systems and in peripheral organs. More importantly, modulating the activity of the endocannabinoid system turned out to hold therapeutic promise in a wide range of disparate diseases and pathological conditions, ranging from mood and anxiety disorders, movement disorders such as Parkinson's and Huntington's disease, neuropathic pain, multiple sclerosis and spinal cord injury, to cancer, atherosclerosis, myocardial infarction, stroke, hypertension, glaucoma, obesity/metabolic syndrome, and osteoporosis, to name just a few. An impediment to the development of cannabinoid medications has been the socially unacceptable psychoactive properties of plant-derived or synthetic agonists, mediated by CB_1 receptors. However, this problem does not arise when the therapeutic aim is achieved by treatment with a CB_1 receptor antagonist, such as in obesity, and may also be absent when the action of endocannabinoids is enhanced indirectly through blocking their metabolism or transport. The use of selective CB_2 receptor agonists, which lack psychoactive properties, could represent another promising avenue for certain conditions. The abuse potential of plant-derived cannabinoids may also be limited through the use of preparations with controlled composition and the careful selection of dose and route of administration. The growing number of preclinical studies and clinical trials with compounds that modulate the endocannabinoid system will probably result in novel therapeutic approaches in a number of diseases for which current treatments do not fully address the patients' need. Here, we provide a comprehensive overview on the current state of knowledge of the endocannabinoid system as a target of pharmacotherapy.

I. Introduction

Marijuana, or cannabis, is the most widely used illicit drug in Western societies and also the one with the longest recorded history of human use. The popularity of marijuana as a recreational drug is due to its ability to alter sensory perception and cause elation and euphoria, most vividly described by the 19th century French poet, Charles Baudelaire, in his book *Les Paradis Artificiels* (Iversen, 2000). However, the ability of extracts of the hemp plant (*Cannabis sativa*) to cause a variety of medicinal effects unrelated to its psychoactive properties had been recognized as early as the third millennium BC, when Chinese texts described its usefulness in the relief of pain and cramps (Mechoulam, 1986). In ancient India, the anxiety-relieving effect of bhang (the Indian term for marijuana ingested as food) had been recorded more than 3000 years ago. The use of cannabis or hashish as a psychoactive substance reached Europe and the Americas through the Arab world in the 19th century. During the same period, cannabis extracts had gained widespread use for medicinal purposes until 1937, when concern about the dangers of abuse led to the banning of marijuana for further medicinal use in the United States. The rather turbulent history of marijuana and the recent resurgence of interest in its medicinal properties have been the subject of excellent reviews (Mechoulam, 1986; Iversen, 2000; Di Marzo et al., 2004; Howlett et al., 2004; Pertwee, 2005a; Piomelli, 2005; Di Marzo and Petrocellis, 2006; Mackie, 2006; Pagotto et al., 2006). Added to this interest is the emergence of the endocannabinoid system, offering not only new insights into the mechanisms underlying the therapeutic actions of plant-derived phytocannabinoids but also novel molecular targets for pharmacotherapy. In this overview, we will briefly summarize current thoughts about the role of endocannabinoids in a given physiological or pathological process and then survey attempts to exploit this role for therapeutic gain.

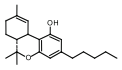
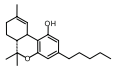
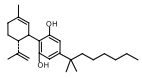
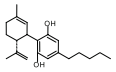
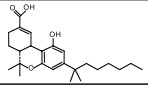
II. The Pharmacology of Cannabinoids

A. Cannabinoid Receptors and Ligands

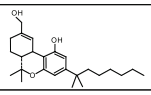
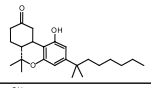
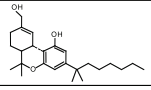
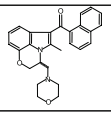
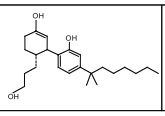
Up until the last two decades, marijuana research was a rather esoteric field, of interest to a small number of scientists. A contributory factor was the highly lipophilic nature of the biologically active ingredients, which led to the notion that marijuana elicits its effects nonspecifically by perturbing membrane lipids (Lawrence and Gill, 1975). The first important breakthrough that ultimately led to a rejection of this concept was the identification by Gaoni and Mechoulam (1964) of the correct chemical structure of the main psychoactive ingredient of marijuana, Δ^9-tetrahydrocannabinol (THC[1]), and the subsequent demonstration that bioactivity resides in the *l*-stereoisomer of this compound (Mechoulam and Gaoni, 1967), which is one of approximately 60 cannabinoids present in the plant (Dewey, 1986). This discovery stimulated the generation of a whole range of synthetic analogs in the 1970s that included not only compounds structurally similar to phytocannabinoids (Fig. 1A) but also analogs with different chemical structures, including classic and nonclassic cannabinoids and aminoalkylindoles (Fig. 1B) (Howlett et al., 2002), as well as the subsequently discovered endogenous arachidonic acid derivatives or endocannabinoids (Fig. 1C), which are discussed in more detail below. Studies of the biological effects of THC and its synthetic analogs revealed strict structural selectivity (Hollister, 1974) as well as stereoselectivity (Jones et al., 1974), telltale signs of drug-receptor interactions. Definitive evidence for the existence of specific cannabinoid receptors was followed soon by the demonstration of high-affinity, saturable, stereospecific binding sites for the synthetic cannabinoid agonist [^{3}H]CP-55,940 in mouse brain plasma membranes, which correlated with both the in vitro inhibition of adenylate cyclase and the in vivo analgesic effect of the compound (Devane et al., 1988). The availability of a radioligand also allowed the mapping of cannabinoid receptors in the brain by receptor autoradiography (Herkenham et al., 1991b). This mapping turned out to be of key importance in the subsequent identification of an orphan G protein-coupled receptor (GPCR) as the brain receptor for cannabinoids (Matsuda et al., 1990), later named CB_1 receptor, based on the overlapping regional distribution of the mRNA for this GPCR and [^{3}H]CP-55,940 binding sites. CB_1 receptors are the most abundant receptors in the mammalian brain but are also present at much lower concentrations in a variety of peripheral tissues and cells. A second cannabinoid GPCR, CB_2, is expressed primarily in cells of the immune and hematopoietic systems (Munro et al., 1993) but recently were found to be present in the brain (Van Sickle et al., 2005; Gong et al., 2006), in nonparenchymal

[1] Abbreviations: THC or Δ^9-THC, Δ^9-tetrahydrocannabinol; CP-55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; GPCR, G protein-coupled receptor; CB_1 or CB_2, cannabinoid 1 or 2; CBD, cannabidiol; SR141716, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride (rimonabant); AM251, *N*-(piperin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; $TRPV_1$ or VR_1, transient receptor potential vanilloid 1 or vanilloid 1; WIN 55,212-2, *R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; HU-210, Δ^8-tetrahydrocannabinol dimethyl heptyl; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; 2-AG, 2-arachidonoylglycerol; NAPE; *N*-arachidonoyl phosphatidylethanolamide; PE, phosphatidylethanolamine; PL, phospholipase; DAG, diacylglycerol; FAAH, fatty acid amide hydrolase; UCM707, *N*-(3-furanylmethyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide; LY2318912, 5-(4-azido-3-iodo-benzoylaminomethyl]-tetrazole-1-carboxylic acid dimethylamide; MGL, monoacylglyceride lipase; DSI, depolarization-induced suppression of inhibition; SR144528, *N*-((1*S*)-endo-1,3,3-trimethyl bicyclo heptan-2-yl]-5-(4chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide); NPY, neuropeptide Y; MCH, melanin concentrating hormone; α-MSH, α-melanocyte-stimulating hormone; CRH, corticotropin-releasing hormone; CART, cocaine- and amphetamine-related transcript; AMPK, AMP-activated protein kinase; ACC1, acetyl CoA carboxylase-1; SREBP1c, sterol response element binding protein 1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CNS, central nervous system; HIV, human immunodeficiency virus; LPS, lipopolysaccharide or endotoxin; TNF-α, tumor necrosis factor-α; IL, interleukin; CXCL, CXC chemokine ligand; NMDA receptor, *N*-methyl-D-aspartate receptor; HU-211, dexanabinol; TBI, traumatic brain injury; BAY 38-7271, (−)-(*R*)-3-(2-hydroxymethylindanyl-4-oxy)phenyl-4,4,4-trifluoro-1-sulfonate; MCAo, middle cerebral artery occlusion; GABA, gamma-aminobutyric acid; GPe or GPi, external or internal globus pallidus; HD, Huntington's disease; HPA axis, hypothalamic-pituitary-adrenal axis; HU-211, dexanabinol; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; I/R, ischemia reperfusion; KA, kainic acid; LID, levodopa-induced dyskinesia; methyl-D-aspartate receptor; NO, nitric oxide; PD, Parkinson's disease; LY320135, [6-methoxy-2-(4-methoxyphenyl)benzo[*b*]-thien-3-yl][4-cyanophenyl] methanone; MS, multiple sclerosis; SCI, spinal cord injury; EAE, experimental autoimmune encephalomyelitis; JWH-133, 1,1-dimethylbutyl-1-deoxy-Δ^9-tetrahydrocannabinol; PEA, palmitoylethanolamide; ACEA, arachidonyl-2′-chloroethylamide/(all *Z*)-*N*-(2-cycloethyl)-5,8,11,14-eicosatetraenamide; JWH-015, (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone; OMDM1, (*R*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine; OMDM2, (*S*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine; SNr, substantia nigra pars reticulata; LID, levodopa-induced dyskinesia; GPe or GPi, external or internal globus pallidus; HD, Huntington's disease; ALS, amyotrophic lateral sclerosis; AM404, *N*-(4-hydroxyphenyl)-eicosa-5,8,11,14-tetraenamide; VDM11, *N*-(4-hydroxy-2-methylphenyl) arachidonoyl amide; AM374, palmitylsulfonyl fluoride; TS, Gilles de la Tourette's syndrome; AD, Alzheimer's disease; Aβ, β-amyloid; HPA, hypothalamic-pituitary-adrenal; URB597, cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester; 5-HT, 5-hydroxytryptamine (serotonin); VTA, ventral tegmental area; nAc, nucleus accumbens; CPP, conditioned place preference; MDMA, 3,4-methylenedioxymethamphetamine (Ecstasy); SHR, spontaneously hypertensive rat(s); WKY, Wistar-Kyoto; AM281, *N*-(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1-*H*-indol-3-yl(4-methoxyphenyl)-methanone; IBD, inflammatory bowel disease; PRS-211,092, [(+)-(6a*S*,10a*S*)-6,6-dimethyl-3-(1,1-dimethylheptyl)-1-hydroxy-9-(1*H*-imidazol-2-ylsulfanylmethyl]-6a-,7,10,10a-tetrahydro-6*H*-dibenzo[*b*,*d*]pyran; RA, rheumatoid arthritis; HU-320, cannabidiol-dimethylheptyl-7-oic acid; HU-308, (+)-(1-a*H*,3*H*,5a*H*)-4-[2,6-dimethoxy-4-(1,1-dimethylheptyl)phenyl]-6,6-dimethylbicyclo[3.1.1]hept-2-ene-2-carbinol.

A. Phytocannabinoids and their analogs

Δ^9-tetrahydrocannabinol (Δ^9-THC) 	$CB_1 \approx CB_2$ agonist	Felder et al., 1995 Schowalter et al., 1996 Rinaldi-Carmona et al., 1994 Rhee et al., 1997
Cannabivarin (Cannabivarol, CBV) 	$CB_1 \approx CB_2$ antagonist	Thomas et al., 2005
(-)-5'-(1,1-dimethylheptyl) cannabidiol (DMH-CBD) 	$CB_1 \approx CB_2$ agonist inhibition of AEA uptake	Bisogno et al., 2001
(-)-Cannabidiol (CBD) 	no activity at CB_1 or CB_2 antagonism of non-CB_1 or non-CB_2 modulator of α_1-adrenoreceptor inhibition af AEA uptake and metabolism	Schowalter et al., 1996 Járai et al., 1999 Pertwee et al., 2002 Bisogno et al., 2001
Ajulemic acid (AJA, CT-3, IP-751) 	$CB_1 \approx CB_2$ agonist	Dyson et al., 2005

B. Synthetic cannabinoids

Classical		
(-) HU-210 	$CB_1 \approx CB_2$	Felder et al., 1995 Schowalter et al., 1996
Nabilone 	$CB_1 \approx CB_2$	Gareau et al., 1996
(+) HU-211 (dexanabinol) 	no activity at CB_1 or CB_2 noncompetitive NMDA antagonist	Schowalter et al., 1996 Bar-Joseph et al., 1994
Non-Classical		
WIN 55,212-2 	$CB_1 \approx CB_2$ agonist	Rinaldi-Carmona et al., 1994 Hillard et al., 1999 Felder et al., 1995
Aminoalkylindol		
(-) CP55940 	$CB_1 \approx CB_2$ agonist	Rinaldi-Carmona et al., 1994 Hillard et al., 1999 Felder et al., 1995 Ross et al., 1999

C. Endogenous cannabinoids (eicosanoids)

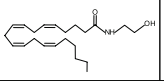
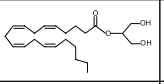
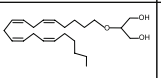
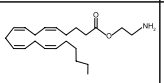
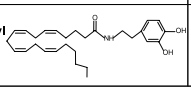
Anandamide (AEA) 	$CB_1 >> CB_2$ agonist $TRPV_1$ agonist	Mechoulam et al., 1995 Khanolkar et al., 1996 Schowalter et al., 1996 Felder et al., 1995 Zygmunt et al., 1999
2-Arachidonoyl glycerol (2-AG) 	$CB_1 \approx CB_2$ agonist	Mechoulam et al., 1995 Ben-Shabat et al., 1998
2-Arachidonoyl glycerol ether 	$CB_1 >> CB_2$ agonist	Hanus et al., 2001
O-Arachidonoyl ethanolamine (virodhamine) 	$CB_1 >> CB_2$ agonist	Porter et al., 2002
N-Arachidonoyl dopamine 	$CB_1 >> CB_2$ agonist $TRPV_1$ agonist	Bisogno et al., 2000 Huang et al., 2002

FIG. 1. The chemical structure and pharmacological activity of selected plant derived (A), synthetic (B), and endogenous cannabinoids (C).

cells of the cirrhotic liver (Julien et al., 2005), in the endocrine pancreas (Juan-Pico et al., 2005), and in bone (Karsak et al., 2004; Idris et al., 2005; Ofek et al., 2006).

Two splice variants of CB_1 receptors have been also identified: CB_{1A}, which has an altered amino-terminal sequence (Shire et al., 1995), and CB_{1B}, which has an in-frame deletion of 33 amino acids at the amino terminus (Ryberg et al., 2005). The mRNAs of both splice variants are expressed at much lower levels than the CB_1 mRNA and, although the receptors expressed from the cDNAs have unique pharmacology (Ryberg et al., 2005), evidence for their natural expression has not been reported.

An interesting twist on the steric selectivity of cannabinoid receptors has emerged through recent studies of the behaviorally inactive phytocannabinoid (−)-cannabidiol (CBD) and its synthetic analogs, which have negligible affinity for either CB_1 or CB_2 receptors. Paradoxically, some of the synthetic (+)-(+)-stereoisomers of these compounds were found to bind potently to both CB_1 and CB_2 receptors (Bisogno et al., 2001) but to display only peripheral and not centrally mediated cannabinoid-like bioactivity, suggesting that they may act as antagonists rather than agonists at central, but not peripheral, CB_1 receptors (Fride et al., 2005).

Another ligand that displays central versus peripheral selectivity is ajulemic acid, a metabolite of THC that was found to have potent anti-inflammatory and analgesic properties without any overt behavioral or psychoactive effects (Burstein et al., 1992; Dyson et al., 2005; Mitchell et al., 2005). Ajulemic acid was reported to bind to both CB_1 and CB_2 receptors with reasonably high affinity (K_d 100–200 nM) but only to activate the latter (Rhee et al., 1997), which may explain its unique and therapeutically attractive pharmacological profile. A more recent study indicated even higher affinities for CB_1 (K_i 6 nM) and CB_2 receptors (K_i 56 nM) and specified the role of CB_1 in mediating its antihyperalgesic activity in neuropathic pain (Dyson et al., 2005). This article also documented limited brain penetration of ajulemic acid compared with other cannabinoids, which may account for its favorable therapeutic profile. Ajulemic acid also binds to peroxisome proliferator-activated receptor γ receptors with low (micromolar) affinity, which was proposed to account for its effect on adipocyte differentiation (Liu et al., 2003b).

Among the 60 or so cannabinoids present in marijuana, only THC is psychoactive. However, some of the other constituents, such as cannabidiol, have well-documented biological effects of potential therapeutic interest, such as antianxiety, anticonvulsive, antinausea, anti-inflammatory and antitumor properties (Mechoulam et al., 2002c; Grotenhermen, 2004; Vaccani et al., 2005). Cannabidiol does not significantly interact with CB_1 or CB_2 receptors, and its actions have been attributed to inhibition of anandamide degradation or its antioxidant properties (Mechoulam and Hanus, 2002; Mechoulam et al., 2002c), or an interaction with as yet unidentified cannabinoid receptors (see below). Another

marijuana constituent of potential therapeutic interest is tetrahydrocannabivarin (Markus, 1971), which has recently been shown to have CB_1 antagonist properties (Thomas et al., 2005).

In addition to CB_1 and CB_2 receptors, pharmacological evidence has been accumulating over the years to support the existence of one or more additional receptors for cannabinoids (reviewed in Begg et al., 2005). Two of these possibilities have been more extensively explored: an endothelial site involved in vasodilation and endothelial cell migration (Járai et al., 1999; Begg et al., 2003; Mo et al., 2004), and a presynaptic site on glutamatergic terminals in the hippocampus mediating inhibition of glutamate release (Hájos et al., 2001). Responses elicited at both of these sites were reported to survive genetic ablation of CB_1 receptors, yet be sensitive to inhibition by the CB_1 antagonist SR141716 or by pertussis toxin but not by the CB_1 antagonist AM251 (Járai et al., 1999; Hájos and Freund, 2002; Ho and Hiley, 2003; Offertáler et al., 2003; O'Sullivan et al., 2004a,b). However, the two sites are apparently different. The aminoalkylindol WIN 55,212-2 was found to be an agonist and capsazepine an antagonist at the hippocampal (Hájos and Freund, 2002) but not at the endothelial receptor (Wagner et al., 1999; Mukhopadhyay et al., 2002). On the other hand, certain atypical cannabinoids with no affinity for CB_1 or CB_2 receptors behave as agonists (abnormal cannabidiol, O-1602) or antagonists at the endothelial receptor (cannabidiol, O-1918) but not at the hippocampal receptor (Begg et al., 2005). Arachidonoyl-L-serine, an endogenous lipid discovered in rat brain, has been found to be a vasodilator acting at the endothelial cannabinoid receptor (Milman et al., 2006), although its activity at the hippocampal receptor has not yet been evaluated. The existence of this latter receptor has recently been called into question, as the ability of WIN 55,212-2 to suppress the same excitatory synapse as studied by Hájos et al. (2001) was found to be absent in two different strains of CB_1 knockout mice, yet present in their respective wild-type controls (Takahashi and Castillo, 2006). Atypical cannabinoid receptors with pharmacological properties similar to those of the endothelial receptor have been postulated to exist on microglia, where they mediate microglial migration (Walter et al., 2003), and on neurons of the mouse vas deferens (Pertwee et al., 2002, 2005c). Activation of this latter receptor by the CBD analog 7-OH-dimethylheptyl CBD, which is inactive at CB_1, CB_2, or transient receptor potential vanilloid type 1 ($TRPV_1$) receptors, inhibits electrically evoked contractions of the vas deferens, and the effect is selectively inhibited by CBD itself. A brain cannabinoid receptor distinct from CB_1 was also indicated by the ability of anandamide and WIN 55,212-2, but not other agonists, to stimulate GTPγS binding in brain plasma membranes from CB_1 knockout mice (Breivogel et al., 2001).

Of interest are recent findings reported in the patent literature that the orphan receptor GPR-55 (Sawzdargo et al., 1999) recognizes a variety of cannabinoid ligands, but not WIN 55,212-2 (Brown and Wise, 2003; Drmota et al., 2004). However, GPR-55 is apparently not expressed in the vascular endothelium and is sensitive to HU-210 (Drmota et al., 2004), a potent synthetic cannabinoid devoid of vasorelaxant properties (Wagner et al., 1999). Furthermore, it couples to G_{12}/G_{13} and ρ kinase, which have been linked to vasoconstrictor rather than vasodilator responses. This suggests that GPR-55 is not the abnormal cannabidiol-sensitive endothelial receptor. Mice deficient in GPR-55 will help in defining the biological functions of this novel cannabinoid-sensitive receptor.

Anandamide has been found to be an agonist ligand for the $TRPV_1$ ion channel, although its affinity in the low micromolar range is lower than its affinity for CB_1 receptors (reviewed by van der Stelt and Di Marzo, 2004). An in vitro study in rat mesenteric arteries provided evidence that the endothelium-independent component of anandamide-induced vasodilation is mediated via activation of capsaicin-sensitive $TRPV_1$ in sensory nerve terminals. This triggers the release of CGRP, which then dilates the artery by activation of calcitonin gene-related peptide receptors on the vascular smooth muscle (Zygmunt et al., 1999). However, this mechanism does not contribute to the in vivo hypotensive action of anandamide, which is similar in wild-type and $TRPV_1^{-/-}$ mice (Pacher et al., 2004).

Both CB_1 and CB_2 receptors are G protein-coupled receptors. Surprisingly, they share little sequence homology, only 44% at the protein level or 68% in the transmembrane domains, which are thought to contain the binding sites for cannabinoids (Lutz, 2002). Despite this, THC and most synthetic cannabinoids have similar affinities for the two receptors, and only recently did synthetic ligands that discriminate between CB_1 and CB_2 receptors emerge. These include agonists as well as antagonists, as listed in Fig. 2. The development of potent and highly selective CB_1 and CB_2 receptor antagonists (Rinaldi-Carmona et al., 1994, 1998) is particularly noteworthy as it provided critically important tools to explore the physiological functions of endocannabinoids. For example, as it will be discussed later in this review, the appetite-reducing effects of the CB_1 antagonist SR141716 in various rodent models was the first sign to suggest that endocannabinoids may be tonically active orexigenic agents, representing the endogenous counterpart of the "munchies" caused by marijuana smoking.

However, these antagonists, as well as most of the other CB_1 and CB_2 antagonists developed to date, have inverse agonist properties (Bouaboula et al., 1997, 1999), so their effects do not necessarily reflect reversal of the tonic action of an endocannabinoid. For this reason, the development of CB_1 and CB_2 receptor-deficient mouse strains (Ledent et al., 1999; Zimmer et al., 1999;

A. Cannabinoid receptor agonists

CB_1 receptor selective	K_i (nM) for CB_1	K_i (nM) for CB_2	Reference
R-(+)-methanandamide	17.9 20 28.3	868 815 868	Lin et al., 1998 Khanolkar et al., 1996 Goutopoulos et al., 2001
Arachidonoyl 2′-chloroethylamide (ACEA)	1.4 5.29	>2,000 195	Hillard et al., 1999 Lin et al., 1998
O-1812	3.4	3,870	Di Marzo et al., 2001a
2-Arachidonoyl glycerol ether	21.2	>3,000	Hanus et al., 2001
CB_2 receptor selective			
JWH 015	383	13.8	Showalter et al., 1996
JWH 133	677	3.4	Huffman et al., 1999
AM 1241	280	3.4	Ibrahim et al., 2003
HU-308	>10,000	22.7	Hanus et al., 1999

B. Cannabinoid receptor antagonists

CB_1 receptor selective	K_i (nM) for CB_1	K_i (nM) for CB_2	Reference
SR 141716	11.8 12.3 5.6 1.8	13,200 702 >1,000 514	Felder et al., 1998 Schowalter et al., 1996 Rinaldi-Carmona et al., 1994 Ruiu et al., 2003
AM251	7.49	2,290	Lan et al., 1999b
AM281	12	4,200	Lan et al., 1999a
LY320135	141	14,900	Felder et al., 1998
CB_2 receptor selective			
SR 144528	437 >10,000 70 50.3	0.60 5.8 0.28 1.99	Rinaldi-Carmona et al., 1998 Ross et al., 1999 Ruiu et al., 2003 Iwamura et al., 2001
AM630	5,152	31.2	Ross et al., 1999

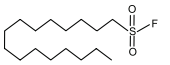
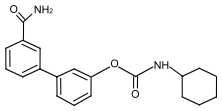
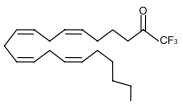
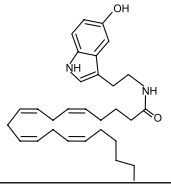
FIG. 2. Selective agonists (A) and antagonists (B) of CB_1 and CB_2 receptors.

Buckley et al., 2000; Marsicano et al., 2002b; Robbe et al., 2002) was similarly important, as the use of these animals in combination with receptor antagonists can reinforce the putative regulatory roles of endocannabinoids. More recently, the development of conditional mutant mice that lack the expression of CB_1 receptors only in certain types of neurons represents another milestone, as it allows linking of specific neuronal populations with a well-defined cannabinoid-modulated behavior (Marsicano et al., 2003).

B. Cannabinoid Receptor Signaling

CB_1 and CB_2 receptors couple primarily to the $G_{i/o}$ subtypes of G protein, and their signaling is remarkably complex. Although coupling to adenylate cyclase through $G_{i/o}$ usually results in inhibition of cyclase activity through the release of $G_{i\alpha}$ isoforms, cannabinoids can also stimulate isoforms 2, 4, or 7 of adenylate cyclase via the release of $\beta\gamma$ subunits (Rhee et al., 1998). Activation of adenylate cyclase also occurs when CB_1 and dopamine D_2 receptors are simultaneously activated (Glass and Felder, 1997), probably as a result of heterodimerization of these two types of receptors (Kearn et al., 2005). Although direct evidence for the coupling of CB_1 receptors to $G_{q/11}$ had until recently been lacking (Howlett, 2004), the agonist WIN 55,212-2, but not other cannabinoids, was recently reported to increase intracellular calcium in cultured hippocampal neurons and in human embryonic kidney 293 cells via coupling to $G_{q/11}$ proteins (Lauckner et al., 2005). Receptor dimerization may facilitate such coupling, which may account for CB_1-mediated mobilization of intracellular calcium in NG108-15 neuroblastoma glioma cells (Sugiura et al., 1999). Cannabinoids can also inhibit different types of calcium channels (Mackie and Hille, 1992; Gebremedhin et al., 1999) and activate certain potassium channels (Mackie et al., 1995) via G protein $\beta\gamma$ subunits (Ikeda, 1996). Cannabinoids can activate members of all three families of multifunctional mitogen-activated protein kinases, including p44/42 MAP kinase (Wartmann et al., 1995; Davis et al., 2003), p38 kinase (Liu et al., 2000; Derkinderen et al., 2001), and JUN-terminal kinase (Liu et al., 2000; Rueda et al., 2000) and activate the phosphatidylinositol-3-kinase pathway (Gómez Del Pulgar et al., 2002a). These effects could be via G protein activation (Galve-Roperh et al., 2002; Davis et al., 2003) or pathways independent of G proteins via other adaptor proteins (Sánchez et al., 2001b). Another G protein-independent pathway activated by cannabinoids involves G protein-coupled receptor kinase-3 and β-arrestin-2, which are required for desensitization, but not for internalization, of CB_1 receptors, and the related development of tolerance (Jin et al., 1999). Cannabinoids can also regulate the activity of phosphatases, as exemplified the CB_1-mediated regulation of calcineurin (protein phosphatase 2b) (Cannich et al., 2004) or the activation of mitogen-activated protein kinase phosphatase 1, which plays an important role in the anti-inflammatory action of anandamide (Eljaschewitsch et al., 2006).

Different structural classes of cannabinoid receptor agonists have the unique ability to activate different

FAAH inhibitors (FAAHI)		K_d (nM) for CB_1	CB_2	FAAHI	ACUI	$TRPV_1$	MGL	Reference
Palmitylsulphonyl fluoride (AM 374)		520		13-50				Deutsch et al., 1997a
URB 597				113 0.5-4.6		>1 mM	>1 mM >30 mM	Lichtman et al., 2004a Kathuria et al., 2003
Arachidonyl Trifluoromethyl Ketone (AATFMK)		0.65-2.5		900 1,000 4,000			2,500 30,000	Deutsch et al., 1997a,b Ueda et al., 1995 Beltramo et al., 1997 Koutek et al., 1994 Dinh et al., 2002b Goparaju et al., 1999
Arachidonoyl Serotonin		inactive		560-1200				Bisogno et al., 1998

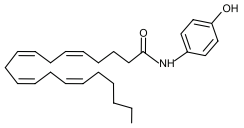
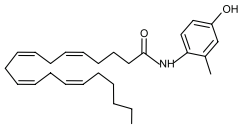
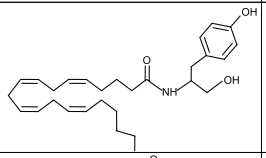
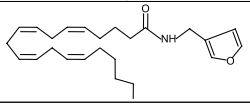
AEA cellular uptake inhibitors (ACUI)		CB_1	CB_2	FAAHI	ACUI	$TRPV_1$	MGL	Reference
AM404				inhibit	4,000	agonist		Beltramo et al., 1997 De Petrocellis et al., 2000 Jarrahian et al., 2000
VDM 11				inhibit	10,000			De Petrocellis et al., 2000
OMDM 1		12,000			2,4000	>50,000	>10,000	Ortar et al., 2003 Fowler et al., 2004
UCM 707			67	30,000	80 25,000			Lopez-Rodriguez et al., 2001; 2003 Fowler et al., 2004

FIG. 3. The structure and pharmacological specificity of inhibitors of FAAH and of endocannabinoid membrane transport.

signaling cascades which, in turn, influences agonist efficacy. Using an in situ receptor/G protein reconstitution technique, CB_1 receptors were found to efficiently couple and activate both G_i and G_o, whereas CB_2 receptors only activated G_o. Furthermore, the efficacy of a given agonist was different whether CB_1 receptors coupled to G_i or G_o, demonstrating agonist-selective G protein signaling (Glass and Northup, 1999). Prather et al. (2000) found that the aminoalkylindol agonist WIN 55,212-2 activated different $G_{i\alpha}$ subunits with markedly different potencies. Even more striking is the recent finding that demonstrates cannabinoid agonist-selective activation of different $G_{i\alpha}$ subunits (Mukhopadhyay and Howlett, 2005). A possible practical implication of such findings is that unique therapeutic profiles may be achieved through the use of different agonists for the same receptor, and such profiles may differ from one target tissue to the other, depending on the pattern of G protein subunit expression.

At least part of this agonist selectivity in G protein activation may be related to the existence of distinct binding sites on CB_1 receptors for different classes of ligands, as documented by site-directed mutagenesis and molecular modeling studies (see Reggio, 2003). These studies indicate that a K3.28A mutation in the third transmembrane domain caused a more than 1000-fold loss in affinity and loss of efficacy for anandamide and nonclassic cannabinoids, without affecting the affinity for WIN 55,212-2 (Song and Bonner, 1996). In contrast, mutations at different sites in the third, fifth, and

sixth transmembrane helices (F3.36A, W5.43A, and W6.48A) affected the binding of WIN 55,212-2 and SR141716, but not anandamide (McAllister et al., 2003).

Another important feature of cannabinoid signaling in the brain is the lack of correlation between the density of CB_1 receptors in a given brain region and the efficiency of receptor coupling, as determined by GTPγS binding (Breivogel et al., 1997), which may explain why functionally important responses can be triggered in brain regions with very sparse CB_1 receptor expression, such as the brainstem (Rademacher et al., 2003) or the hypothalamus (Jamshidi and Taylor, 2001). Selley et al. (2001) have shown that the reduction in CB_1 receptor density in CB_1 heterozygote mice was compensated for by an increase in receptor/G protein coupling efficiency for some, but not other, agonists. Although the underlying mechanisms for such compensation are not clear, differences in the degree of receptor multimerization (Mackie, 2005), or changes in signal amplification are possibilities. Recent observations indicate that a considerable proportion of the psychomotor effect of cannabinoids can be accounted for by a signaling cascade in striatal projection neurons involving protein kinase A-dependent phosphorylation of DARPP-32, achieved via modulation of dopamine D_2 and adenosine A_{2A} transmission (Andersson et al., 2005). This represents a unique form of amplification of CB_1 signaling, as phosphorylation of DARPP-32 at Thr-34 amplifies downstream signaling via inhibition of protein phosphatase-1 (Greengard, 2001). It would be interesting to test whether the efficiency of CB_1 coupling to DARPP-32 is affected by cellular receptor density.

C. Endocannabinoids

The existence of specific receptors in mammalian cells that recognize a plant-derived substance rekindled the question raised two decades earlier, after brain receptors for morphine had been first described, i.e., is there an endogenous ligand? A positive answer was provided in 1992 by the report by Devane et al. describing the isolation from porcine brain of the lipid arachidonoyl ethanolamide, named anandamide, which bound to the brain cannabinoid receptor with reasonably high affinity and mimicked the behavioral actions of THC when injected into rodents (Devane et al., 1992). Three years later a second endocannabinoid, 2-arachidonoylglycerol (2-AG), was discovered independently by Mechoulam et al. (1995) and Sugiura et al. (1995). Since then, a number of related endogenous lipids with endocannabinoid-like activity have been reported (Fig. 1c), but follow-up studies about biosynthesis, cellular transport, metabolism, and biological function have focused on anandamide and 2-AG, with much less information available about the other compounds with endocannabinoid-like properties. The biochemical aspects of endocannabinoids have been recently reviewed by Bisogno et al. (2005).

Anandamide is a partial or full agonist of CB_1 receptors, depending on the tissue and biological response measured. Although it also binds CB_2 receptors, it has very low efficacy and may act as an antagonist (Gonsiorek et al., 2000). The in vivo biosynthesis of anandamide (Fig. 4) is believed to occur through the enzymatic hydrolysis catalyzed by a phospholipase D of a membrane lipid precursor, *N*-arachidonoyl phosphatidylethanolamide (NAPE) (Schmid et al., 1983), which itself is generated by the enzymatic transfer of arachidonic acid in the *sn*-1 position in phosphatidylcholine to the amide group of PE (Di Marzo et al., 1994; Cadas et al., 1997). Although a specific transacylase for the latter reaction has not yet been identified, a NAPE-specific PLD has recently been cloned (Okamoto et al., 2004). It is not yet known, however, whether NAPE-PLD is obligatory for the biosynthesis of anandamide, which could make it an attractive target of drug therapy when reduction of tissue anandamide would be of benefit. Indeed, there may be parallel pathways for the generation of anandamide from NAPE. A secretory PLA_2 that can catalyze the hydrolysis of *N*-acyl-PE to *N*-acyl-lysoPE, which is then acted on by a lysoPLD to generate *N*-acyl-ethanolamides, including anandamide, was recently identified in the stomach (Sun et al., 2004). An alternative parallel pathway has been identified in our laboratory in RAW246.7 macrophages. This involves hydrolysis of NAPE to phosphoanandamide by a PLC, followed by dephosphorylation through a phosphatase (Liu et al., 2006). This latter pathway rather than PLD is the target of regulation by bacterial endotoxin, which increases anandamide synthesis in macrophages (Varga et al., 1998; Liu et al., 2003a). The existence of this pathway may also account for the recent finding that anandamide tissue levels are unchanged in NAPE-PLD knockout compared with wild-type mice (Leung et al., 2006).

2-AG is generated from diacylglycerol (DAG) by DAG lipase selective for the *sn*-1 position (Fig. 4). DAG, an intracellular second messenger that activates protein kinase C, can be generated from phosphoinositides by a phosphoinositide-specific PLC or from phosphatidic acid by phosphatidic acid phosphohydrolase (Bisogno et al., 2005). Two DAG lipase isozymes, α and β, have been cloned (Bisogno et al., 2003). In the adult brain they are localized in the postsynaptic plasma membrane, in line with their putative role in generating 2-AG involved in retrograde transmission.

Basal levels of 2-AG in the brain are approximately 2 orders of magnitude higher than the levels of anandamide. Despite this, stimulus-induced release resulting in detectable extracellular levels could be demonstrated only for anandamide and not for 2-AG in an in vivo microdialysis study (Giuffrida et al., 1999). This finding illustrates that, despite growing interest in endocannabinoids and their roles as retrograde neurotransmitters (Wilson and Nicoll, 2002; Chevaleyre et al., 2006), the mechanism of their release is not well understood.

Like prostanoids, endocannabinoids are not stored but generated on demand in response to a depolarization-induced rise in intracellular calcium or activation of various metabotropic receptors (Varma et al., 2001; Kim et al., 2002; Witting et al., 2004; Di et al., 2005a,b). A putative membrane endocannabinoid transporter involved in the cellular uptake of endocannabinoids (see below) may also be involved in their release. This is suggested by the ability of a transport inhibitor to prevent the release of intracellularly applied anandamide (Maccarrone et al., 2000a; Gerdeman et al., 2002).

Anandamide present in the extracellular space is accumulated by neurons and other cells by facilitated diffusion. This process is driven by its transmembrane concentration gradient, is saturable and temperature-dependent, and does not require ATP or sodium ions. Most importantly for the topic of the present review, anandamide uptake is selectively inhibited by a variety of structural analogs, which suggests the existence of a saturable cellular component involved in anandamide transport (Beltramo et al., 1997; Bisogno et al., 1997; Hillard and Jarrahian, 2000; Maccarrone et al., 2000a). However, a specific anandamide transporter protein has yet to be cloned, and it has been proposed that intracellular degradation of anandamide by fatty acid amide hydrolase (FAAH) is sufficient to account for anandamide uptake in long incubation periods (Glaser et al., 2003). Studies with cells isolated from $FAAH^{+/+}$ and $FAAH^{-/-}$ mice did not resolve this issue, as the absence of FAAH was found not to affect anandamide uptake (Fegley et al., 2004) or to reduce it substantially (Ortega-Gutierrez et al., 2004), albeit under different experimental conditions. Nevertheless, a FAAH-independent component of anandamide uptake, inhibited by the compound UCM707, was detected in the latter study, supporting the notion of a protein other than FAAH being involved. This notion is also supported by the emergence of a number of synthetic transport inhibitors, the potencies of which to inhibit anandamide uptake does not correlate with their affinities for CB_1, CB_2, or $TRPV_1$ receptors or their potencies to inhibit FAAH (Fig. 3). However, in view of the important role of FAAH in generating the transmembrane concentration gradient for anandamide, the possibility that a noncatalytic region of FAAH or a FAAH-associated protein may act as anandamide transporter cannot be excluded. Interestingly, the elucidation of the crystal structure of FAAH revealed several channel-like regions in the enzyme, granting it simultaneous access to both the cytosolic and membrane domains (Bracey et al., 2002). Against this possibility, however, is the recent report that the novel, high affinity anandamide transport inhibitor LY2318912 binds with similar K_d and b_{max} values to membranes from HeLa cells devoid of FAAH or transfected with FAAH, pointing to a binding site independent of the FAAH molecule (Moore et al., 2005). Arguments for and against the existence of a bidirectional anandamide transporter have been recently reviewed (Hillard and Jarrahian, 2003; Fowler et al., 2004; McFarland and Barker, 2004; Glaser et al., 2005).

In some in vivo studies, treatment with transport inhibitors unmasked cannabinoid-like tonic effects on pain sensitivity, anxiety-like behaviors, locomotor activity, and muscle spasticity, which is an indication of the potential therapeutic usefulness of such compounds (Moore et al., 2005; Bortolato et al., 2006; La Rana et al., 2006). Similar and more pronounced effects have been reported in response to treatment with FAAH inhibitors, as discussed below.

In contrast to the unsettled status of anandamide transport and a putative transporter protein, the unique role of FAAH in the in vivo degradation of anandamide has been extensively documented (reviewed in McKinney and Cravatt, 2005). Initial evidence for a membrane-associated enzyme in the liver that hydrolyzes *N*-*N*-acyl ethanolamides (Schmid et al., 1985) was followed by the cloning of FAAH (Cravatt et al., 1996) and the identification of its crystal structure in complex with an active site-directed inhibitor (Bracey et al., 2002). The unique role of FAAH in terminating signaling by anandamide was indicated by the phenotype of FAAH knockout mice, which displayed 10 to 15 times elevated levels of anandamide across the brain, supersensitivity to the actions of exogenous anandamide, and the appearance of tonic signaling by endogenous anandamide, resulting in CB_1 receptor-mediated hypoalgesia (Cravatt et al., 2001; Lichtman et al., 2004b), reduced anxiety (Kathuria et al., 2003), antidepressant activity (Gobbi et al., 2006), and lowering of blood pressure in different models of experimental hypertension (Bátkai et al., 2004b). Cravatt et al. (2004) were able to resolve the relative roles of central versus peripheral fatty acid amides by generating mice deficient in FAAH in peripheral tissues only. These mice did not display the hypoalgesia observed in mice with global deficiency in FAAH, but had a similar anti-inflammatory phenotype, indicating that the latter was mediated by elevated fatty acid amides in peripheral tissues (Cravatt et al., 2004). Interestingly, another amidohydrolase catalyzing the same reaction as FAAH but at acidic pH was recently identified and cloned (Tsuboi et al., 2005). This lysosomal enzyme is structurally unrelated to FAAH and is widely distributed in tissues, with highest levels in the lung, and has been recently shown to contribute to the physiological degradation of anandamide in macrophages but not in the brain (Sun et al., 2005).

Although 2-AG is also hydrolyzed by FAAH under in vitro conditions (Goparaju et al., 1998; Lang et al., 1999), in vivo it is not a substrate of FAAH, as indicated by the unchanged brain levels of 2-AG in wild-type and $FAAH^{-/-}$ mice (Osei-Hyiaman et al., 2005a). 2-AG is hydrolyzed in vivo by a monoacylglyceride lipase (MGL) (Dinh et al., 2002a,b; Saario et al., 2004). A study of the ultrastructural distribution of FAAH and MGL revealed

that in the hippocampus, cerebellum, and amygdala, FAAH is located postsynaptically, whereas MGL is localized in presynaptic axon terminals, including terminals of GABAergic interneurons (Gulyas et al., 2004). Correspondingly, functional studies in hippocampus indicate that depolarization-induced suppression of inhibition (DSI) is unaffected by pharmacological blockade of FAAH (Kim and Alger, 2004), but it is potentiated by blocking MGL (Kim and Alger, 2004; Makara et al., 2005), in agreement with an earlier study implicating 2-AG rather than anandamide in synaptic plasticity in the hippocampus (Stella et al., 1997). Further evidence supporting the role of 2-AG as the retrograde transmitter involved in synaptic plasticity is the preferential postsynaptic distribution of the major 2-AG biosynthetic enzyme, diacylglycerol lipase α, in hippocampus and cerebellum (Katona et al., 2006; Yoshida et al., 2006).

However, the behavioral consequences of DSI and its modulation remain unclear: selective knockout of CB_1 receptors from GABAergic interneurons was found to abolish DSI and long-term depression (LTD) of inhibitory synapses, whereas the classic behavioral responses to THC remained unaffected in these animals (Monory et al., 2005). Therefore, at this point it is difficult to predict the potential therapeutic usefulness of selective MGL inhibitors.

III. The Endocannabinoid System as Therapeutic Target in Pathophysiological Conditions

A. Diseases of Energy Metabolism

1. Endocannabinoids and Appetite Regulation. It has been known since antiquity that use of cannabis in its various forms increases appetite, particularly for palatable foods, and can also result in significant weight gain (Donovan, 1845; Berry and Mechoulam, 2002). Following the identification of THC as the main psychoactive principle in marijuana, the appetite-promoting effect of smoked marijuana could be attributed to THC even before the identification of specific cannabinoid receptors (Hollister, 1971; Greenberg et al., 1976). Animal studies also documented the ability of THC to promote food intake, although consistent effects were only seen with relatively low doses (Abel, 1975), most likely because the significant sedation and motor impairment seen with higher doses interferes with the animals' ability to initiate feeding. Variability in the observed changes in THC-induced food intake may also relate to the feeding state of the animal, the orexigenic effect being optimal in presatiated animals with low basal levels of food intake (Williams et al., 1998). After the discovery of specific cannabinoid receptors and the introduction of selective antagonists, the increase in food

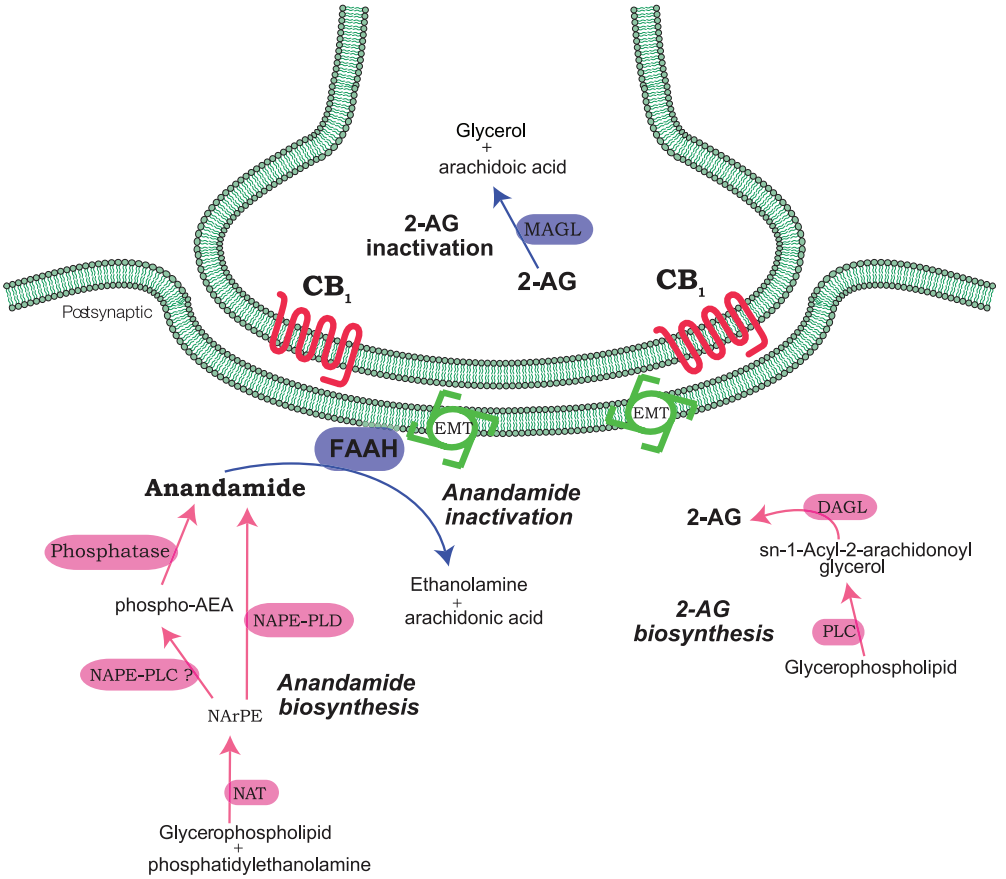


FIG. 4. Schematic representation of the endocannabinoid system in pre- and postsynaptic neurons. The presynaptic terminal is located in the top, whereas the postsynaptic neuron is located in the bottom. EMT, endocannabinoid membrane transporter; MAGL, monoacylglyceride lipase; DAGL, DAG lipase; AEA, anandamide; NArPE, *N*-arachidonyl phosphatidylethanolamine; NAT, *N*-acyltransferase.

intake caused by THC could be linked to CB_1 receptors, as it was blocked by the selective CB_1 antagonist SR141716, but not by the CB_2 antagonist SR144528 (Williams and Kirkham, 2002).

The discovery of endocannabinoids has raised the question of their potential involvement in the physiological control of appetite and energy metabolism. This subject has been the topic of a number of recent reviews (Di Marzo and Matias, 2005; Kirkham, 2005; Sharkey and Pittman, 2005; Pagotto et al., 2006), and only a brief summary is provided here. The first indication of a role for endocannabinoids in appetite control was the documented ability of low doses of anandamide to increase food intake, when administered either systemically (Williams and Kirkham, 1999; Hao et al., 2000) or into the ventromedial hypothalamus (Jamshidi and Taylor, 2001), and this effect could be attributed to stimulation of CB_1 receptors (Williams and Kirkham, 1999). Similar increases in food intake can be elicited by 2-AG administered systemically or into the nucleus accumbens shell region (Kirkham et al., 2002) or into the lateral hypothalamus (Kirkham and Williams, 2001a). Sites for the orexigenic actions of endocannabinoids in both the hypothalamus and the limbic forebrain suggest their involvement in both the homeostatic and hedonic control of eating (Harrold and Williams, 2003; Vickers and Kennett, 2005). Interestingly, endocannabinoid activation of hypothalamic centers, such as the paraventricular nucleus, may also occur indirectly via CB_1 receptors on peripheral afferent nerve terminals (Gomez et al., 2002), most likely located in the gastrointestinal tract. Such an "indirect" pathway is compatible with recent findings that CB_1 mRNA is present in cholecystokinin-containing neurons in the nodose ganglion, where CB_1 mRNA expression is up-regulated by fasting and down-regulated by refeeding (Burdyga et al., 2004).

Studies with antagonists provide more direct support for a regulatory function of endocannabinoids on feeding. Treatment of rats with SR141716 and the closely related CB_1 antagonist AM251 reduced food intake under free-feeding (Arnone et al., 1997; Colombo et al., 1998a; Simiand et al., 1998; Chambers et al., 2003; Shearman et al., 2003) or operant conditions (Freedland et al., 2000; McLaughlin et al., 2003), suggesting antagonism of the tonic orexigenic effect of an endocannabinoid. However, SR141716 and AM251 are inverse agonists (Gifford and Ashby, 1996; Bouaboula et al., 1997), which may be an alternative mechanism by which they reduce food intake.

Definitive evidence for the involvement of endocannabinoids in the control of food intake has been provided through the use of CB_1 receptor-deficient mice. In a study from our laboratory, food-deprived CB_1 knockout mice were found to eat less than their wild-type littermates, and their food intake was unaffected by SR141716 treatment, whereas in wild-type mice SR141716 reduced food intake to the levels seen in the knockout mice (Di Marzo et al., 2001b). Similar findings have been subsequently reported by others (Wiley et al., 2005). This indicates that part of the hunger-induced increase in food intake is mediated by endocannabinoids acting at CB_1 receptors. CB_1 knockout mice are also resistant to overeating caused by neuropeptide Y (NPY) (Poncelet et al., 2003), and SR141716 inhibits the hyperphagia of leptin-deficient mice even in the absence of temporary food deprivation (Di Marzo et al., 2001b). This latter finding suggests that the absence of leptin results in increased endocannabinoid activity. Indeed, hypothalamic levels of endocannabinoids were elevated in leptin-deficient mice and rats and reduced after leptin treatment, suggesting that endocannabinoids are part of the leptin-regulated neural circuitry involved in appetite regulation (Di Marzo et al., 2001b). Endogenous leptin may similarly suppress endocannabinoid levels, as indicated by our recent unpublished findings using mice with obesity induced by a high-fat diet, which have elevated plasma leptin levels proportional to their increased fat mass. Anandamide levels were significantly lower in the obese mice compared with their lean controls in the hypothalamus, limbic forebrain, and amygdala, with no difference in the cerebellum. Furthermore, there was a significant inverse correlation between plasma leptin levels and anandamide levels in the above three brain regions involved in appetite control but not in the cerebellum.

A possible hypothalamic site for an interaction between leptin and endocannabinoids is the lateral hypothalamus, where CB_1 receptors are present in orexin- and melanin-concentrating hormone (MCH)-containing neurons (Cota et al., 2003), which also express functional leptin receptors (Hübschle et al., 2001; Iqbal et al., 2001). These neurons project to dopaminergic neurons in the ventral tegmental area (Fadel and Deutch, 2002), where they modulate the mesolimbic dopaminergic pathway involved in food reward. Thus, they could also represent a site of integration of hypothalamic and extrahypothalamic structures involved in the orexigenic effect of endocannabinoids. The MCH-containing neurons are tonically inhibited by GABAergic interneurons. Jo et al. (2005) recently demonstrated that this inhibitory tone can be suppressed by the depolarization-induced release of endocannabinoids from the MCH neurons and their retrograde activation of presynaptic CB_1 receptors on the GABAergic interneurons. The resulting increase in the activity of MCH neurons may contribute to the in vivo appetitive effect of endocannabinoids. Furthermore, this DSI could be blocked by leptin through inhibition of voltage-gated calcium channels in the MCH neurons, whereas it was increased 6-fold in leptin-deficient mice (Jo et al., 2005), mirroring the changes in hypothalamic endocannabinoid content by leptin and leptin deficiency reported earlier (Di Marzo et al., 2001b). Another hypothalamic site where a leptin/endocannabinoid interaction may occur is the paraventricu-

lar nucleus. At this site, glucocorticoids have been shown to induce endocannabinoid synthesis and endocannabinoid-induced suppression of synaptic excitation via a cAMP-dependent mechanism, and leptin was found to block these effects by a phosphodiesterase 3B-mediated decrease in intracellular cAMP (Malcher-Lopes et al., 2006). These effects may underlie the orexigenic action of glucocorticoids.

Another recent study indicates the importance of lateral hypothalamic orexin neurons in reward-seeking behavior in general (Harris et al., 2005), suggesting that they may also be targets of the effects of endocannabinoids on drug reward (see section III.B.11.). Additionally, cannabinoids can increase the intake of palatable foods by acting at sites in the brainstem (Miller et al., 2004), which also have reciprocal neural connections with forebrain limbic structures (Saper, 2002). From a behavioral point of view, cannabinoids are involved in both the appetitive and consummatory aspects of feeding behavior (Chaperon et al., 1998; Thornton-Jones et al., 2005), in line with their multiple sites of action in the brain. Such multiple sites of action are also indicated by findings that in THC-naive rats, rimonabant suppressed food-maintained operant responses and metabolic activity in the limbic forebrain, measured by 2-deoxyglucose uptake, whereas in rats made tolerant to THC, an additional metabolic inhibition was detected in the hypothalamus (Freedland et al., 2003). Exposure of rats to a palatable diet containing sucrose and condensed milk resulted in down-regulation of CB_1 receptors in limbic structures involved in the hedonic aspects of feeding, but not in the hypothalamus (Harrold et al., 2002). In the hypothalamus, the very low density of CB_1 receptors is offset by their increased coupling (Breivogel et al., 1997), which may be an alternative target of regulation (Basavarajappa and Hungund, 1999; Wang et al., 2003) that needs to be explored.

Within the appetitive neural circuitry, endocannabinoids have been shown to interact with both orexigenic factors such as endogenous opioids, NPY, orexins, and ghrelin, and anorexigenic factors including α-melanocyte-stimulating hormone (α-MSH), corticotropin-releasing hormone (CRH), and the peptide product of the cocaine and amphetamine-related transcript (CART). Inhibition of food intake by opioid μ receptor antagonists and CB_1 receptor antagonists is supra-additive (Kirkham and Williams, 2001b; Rowland et al., 2001; Chen et al., 2004), suggesting a synergism between the endogenous opioid and cannabinoid systems in mediating the reinforcing effect of food (Solinas and Goldberg, 2005). Indeed, CB_1-deficient mice fail to self-administer morphine (Ledent et al., 1999; Cossu et al., 2001) or to release dopamine in the nucleus accumbens in response to morphine (Mascia et al., 1999), suggesting that the site of this synergism is in the mesolimbic dopaminergic pathway, which is involved in both drug and food reward (Le Foll and Goldberg, 2005). The observation that SR141716 inhibits the orexigenic effect of morphine microinjected into the hypothalamic paraventricular nucleus but not the nucleus accumbens shell suggests additional interactions between the two systems, unrelated to the hedonic aspects of feeding (Verty et al., 2003). A further intriguing parallel between the two systems is that opiate μ receptor knockout mice, just as $CB_1^{-/-}$ mice (see below), are resistant to diet-induced obesity (Tabarin et al., 2005).

As for interactions with NPY, the similar effectiveness of SR141716 to inhibit food intake in wild-type and $NPY^{-/-}$ mice indicates that endocannabinoids are unlikely to be the primary compensatory factor that accounts for the lack of a lean phenotype in $NPY^{-/-}$ mice (Di Marzo et al., 2001b). However, anandamide was found to increase and AM251 to decrease depolarization-induced NPY release in rat hypothalamic explants, suggesting that NPY may contribute to the orexigenic effects of cannabinoids (Gamber et al., 2005). A possible role of orexins in the appetitive effects of endocannabinoids is suggested by the finding that coexpression of the CB_1 and orexin 1 receptors results in a marked potentiation of orexin A-induced signaling (Hilairet et al., 2003). An important site of action of the orexigenic peptide ghrelin is the hypothalamic paraventricular nucleus, where its hyperphagic effect can be blocked by SR141716, suggesting that ghrelin may act via the release of endocannabinoids (Tucci et al., 2004). Endocannabinoids, in turn, may be involved in ghrelin release, at least in the periphery, as suggested by an SR141716-induced decrease in plasma ghrelin levels in rats (Cani et al., 2004).

The proopiomelanocortin-derived peptide α-MSH acting at MC-4 melanocortin receptors is part of the leptin-regulated appetitive circuitry as a major anorectic mediator. The observations that SR141716 inhibits the feeding response induced by blocking MC-4 receptors, whereas α-MSH does not affect THC-induced feeding, suggest that CB_1 receptors are downstream from MC-4 receptors and have an obligatory role in α-MSH effects on food intake (Verty et al., 2004). The peptide product of CART is also a tonically active anorectic mediator (Kristensen et al., 1998) and, unlike α-MSH, may be a downstream mediator of the effect of endocannabinoids. Such an arrangement is suggested by the finding that SR141716 loses its ability to reduce food intake in $CART^{-/-}$ mice (Osei-Hyiaman et al., 2005a). Furthermore, mice deficient in FAAH have reduced levels of CART immunoreactivity in various hypothalamic and extrahypothalamic regions involved in appetite control, which is returned to normal levels by chronic SR141716 treatment (Osei-Hyiaman et al., 2005a). These findings suggest that inhibition of CART release by CB_1 activation may be involved in the orexigenic effect of anandamide. Finally, an interaction between endocannabinoids and CRH is indirectly suggested by coexpression of the mRNA for the CB_1 receptor with the mRNA for CRH

(Cota et al., 2003) or the CRH type 1 receptor (Hermann and Lutz, 2005).

2. Endocannabinoids and Peripheral Energy Metabolism. It is generally accepted that energy intake and utilization are regulated in a coordinated fashion, and factors involved in the central regulation of appetite may also affect peripheral energy metabolism (Seeley and Woods, 2003). The first indirect indication that cannabinoids may affect energy homeostasis through a mechanism other than food intake came from a study of marijuana smokers tested in a hospital inpatient setting (Greenberg et al., 1976). In this study, the marijuana-induced increase in caloric intake leveled off after a few days, whereas weight gain continued throughout the rest of the 21-day observation period, suggesting independent effects on appetite and peripheral energy metabolism. After the introduction of SR141716 as the first selective CB_1 receptor antagonist (Rinaldi-Carmona et al., 1994), a similar conclusion was reached in normal rats treated with SR141716 for 14 days. Tolerance to the anorectic effect of SR141716 developed within 5 days, whereas the reduction in body weight was maintained throughout the treatment period (Colombo et al., 1998a). Later, similar observations were reported in mice with diet-induced obesity, in which food intake was reduced transiently whereas the reduction in body weight was maintained when the animals were chronically treated with SR141716 (Ravinet Trillou et al., 2003) or AM251 (Hildebrandt et al., 2003). These results suggested that factors other than appetite must be involved in the weight-reducing effect of CB_1 antagonists.

Peripheral targets of endocannabinoids include adipocytes, which express CB_1 receptors (Bensaid et al., 2003; Cota et al., 2003). Stimulation of CB_1 receptors on adipocytes can affect lipid metabolism through regulating the level of adiponectin production (Bensaid et al., 2003), by increasing lipoprotein lipase activity (Cota et al., 2003), or by inhibiting AMP-activated protein kinase (AMPK) (Kola et al., 2005), which leads to increased lipogenesis and decrease in fatty acid β-oxidation through reducing the phosphorylation and thus disinhibiting acetyl CoA carboxylase-1 (ACC1), the rate-limiting enzyme in fatty acid synthesis. The work by Cota et al. (2003) provided the first clear evidence of peripheral metabolic targets of endocannabinoids in vivo in a mouse model of diet-induced obesity. By careful analysis of body composition, they were able to establish the lean phenotype of CB_1-deficient mice that had escaped earlier attention. Furthermore, the use of a pair-feeding paradigm revealed that hypophagia accounts for the lean phenotype only in young and not in adult animals, which clearly indicated the involvement of peripheral metabolic target(s) in the latter. The additional documentation of functional CB_1 receptors in primary cultured adipocytes and their role in regulating lipogenesis provided one of the likely peripheral targets for the anabolic effects of endocannabinoids. The lean phenotype of $CB_1^{-/-}$ mice in this study was more prominent in male than in female animals, which could suggest that endocannabinoid regulation of adiposity may be subject to modulation by sex hormones.

Although earlier studies failed to detect CB_1 receptors in the liver, more recently they have been identified in the mouse liver using a combination of methods including reverse transcription-polymerase chain reaction, in situ hybridization, immunohistochemistry, and Western blotting. In the same study, treatment of mice with the cannabinoid agonist HU-210 increased de novo lipogenesis and the expression of the transcription factor sterol regulatory element binding protein 1c (SREBP1c) as well as of its targets, ACC1 and fatty acid synthase (Osei-Hyiaman et al., 2005b). The role of CB_1 receptors in these effects was indicated by the ability of SR141716 to block them and by their absence in CB_1 knockout mice (Osei-Hyiaman et al., 2005b). The hepatic lipogenic pathway may be also directly activated through a cannabinoid-induced decrease in AMPK phosphorylation and activity in the liver (Kola et al., 2005). CB_1 receptors have been also detected in rat hepatocytes (Michalopoulos et al., 2003), in whole mouse liver (Biecker et al., 2004), and in rat and human hepatic stellate cells (Siegmund et al., 2005; Teixeira-Clerc et al., 2006).

Fatty acid metabolism in hypothalamic neurons acts as a sensor of nutrient availability (Obici et al., 2003), and its pharmacological modulation influences food intake (Kim et al., 2004). CB_1 activation was reported to increase *SREBP1c* and *FAS* gene expression in the hypothalamus, and the increased expression of these genes by fasting/refeeding (Paulaskis and Sul, 1988) could be inhibited by SR141716 treatment at the beginning of the refeeding period, which also reduced food intake (Osei-Hyiaman et al., 2005b). Although fatty acid synthesis was not measured directly in the hypothalamus, these findings suggest that the increase in food intake after fasting may involve a CB_1-mediated modulation of the fatty acid synthetic pathway. Modulation of AMPK activity by cannabinoids was documented not only in liver and adipose tissue but also in hypothalamus (Kola et al., 2005), where it has been linked to appetite control (Minokoshi et al., 2004). Thus, the AMPK/ACC1/FAS pathway may represent a common molecular pathway involved in both the central appetitive and the peripheral metabolic effects of endocannabinoids.

Because total caloric intake is similar in wild-type and $CB_1^{-/-}$ mice on a high-fat diet (Ravinet Trillou et al., 2004; Osei-Hyiaman et al., 2005b), the resistance of CB_1-deficient mice to diet-induced obesity must be associated with increased energy expenditure. Exposing wild-type C57BL6/J mice to a high-fat diet decreases energy expenditure, as documented by indirect calorimetry (Hu et al., 2004), which may account for the increase in feed efficiency observed in such animals, whereas in $CB_1^{-/-}$ mice feed efficiency was unaffected by a high-fat diet (Osei-Hyiaman et al., 2005b). This

suggests that the high-fat diet-induced decrease in energy expenditure is mediated by endocannabinoid activation of CB_1 receptors. Accordingly, HU-210 treatment of wild-type mice decreased and SR141716 treatment increased the activity of carnitine palmitoyl transferase-1, the rate-limiting enzyme in fatty acid β-oxidation (D. Osei-Hyiaman and G. Kunos, unpublished observations).

One of the factors involved in this effect in vivo could be adiponectin, the adipocyte-derived hormone that promotes fatty acid β-oxidation (Yamauchi et al., 2002). Indeed, exposure to a high-fat diet resulted in a significant decline in plasma adiponectin in wild-type but not in $CB_1^{-/-}$ mice (Osei-Hyiaman et al., 2005a), and CB_1 receptor activation in isolated adipocytes was found to suppress adiponectin expression (Perwitz et al., 2005; Matias et al., 2006). Expression of the thermogenic uncoupling protein-1 was also down-regulated by CB_1 activation, whereas the expression of the insulin-mimetic adipokine visfatin was increased (Perwitz et al., 2005). Conversely, rimonabant increases adiponectin secretion by adipocytes (Bensaid et al., 2003) and adiponectin plasma levels in obese human subjects (Després et al., 2005), which should lead to increased lipid β-oxidation and thermogenesis in vivo. Chronic treatment of *ob/ob* mice with SR141716 increased thermogenesis, as indicated by increased oxygen consumption at a thermoneutral temperature measured by whole body calorimetry (Liu et al., 2005). Glucose uptake, subsequently measured in the isolated soleus muscle of these animals, was significantly increased in the SR141716-pretreated group. A similar effect in humans may account for the increased glucose tolerance observed in obese patients treated with rimonabant (Van Gaal et al., 2005). These observations could suggest the presence of CB_1 receptors in skeletal muscle, which was recently documented (Pagotto et al., 2006). Alternatively, increased glucose tolerance may be secondary to an effect of SR141716 on CB_1 receptors in the liver. It has been proposed that increased lipid synthesis in the liver may produce insulin resistance in other tissues such as muscle (McGarry, 1992), and CB_1 receptor activation has been shown to contribute to the development of hepatic steatosis in diet-induced obesity (Osei-Hyiaman et al., 2005b). Endocannabinoids may also influence insulin secretion directly in islet β-cells via CB_1 (Matias et al., 2006) or CB_2 receptors (Juan-Pico et al., 2005).

The ability of rimonabant to increase energy expenditure may not be limited to an effect on adiponectin secretion, as indicated by an analysis of the effect of rimonabant treatment on gene expression profiles in lean and diet-induced obese mice as well as $CB_1^{-/-}$ mice (Jbilo et al., 2005). Rimonabant-induced decreases in body weight and adipose tissue mass in obese mice was accompanied by a near-complete reversal of obesity-induced changes in the expression of a wide range of genes. These included genes involved in adipocyte differentiation, lipolysis, generation of futile cycles, and glycolysis. These broad-based targets may underlie the ability of rimonabant to correct symptoms of the metabolic syndrome, as discussed below. They also raise the intriguing possibility that if a CB_1 antagonist that does not cross the blood-brain barrier were available, it could be effective in the treatment of the metabolic syndrome without the risk of adverse CNS side effects (Horvath, 2006).

3. Obesity and Associated Metabolic Abnormalities.

Genetic manipulation of the expression of endogenous proteins has been instrumental in uncovering their regulatory role in normal and pathological phenotypes. When CB_1 knockout mice were first introduced, no change in body mass or feeding pattern had been noted (Ledent et al., 1999; Zimmer et al., 1999). However, in a subsequent study, CB_1 knockout mice were found to have a life-long, small, but significant, weight deficit compared with their wild-type littermates, which could be attributed to a selective deficit in adipose tissue mass (Cota et al., 2003) and was confirmed by others (Ravinet Trillou et al., 2004; Osei-Hyiaman et al., 2005b). Parallel to their decreased fat mass, $CB_1^{-/-}$ mice have lower plasma leptin levels and an increased sensitivity to the anorectic effect of exogenous leptin (Ravinet Trillou et al., 2004).

The possibility that an increase in the activity of the endocannabinoid system may contribute to at least some forms of obesity was suggested by three sets of findings. First, CB_1 antagonists were significantly more efficacious in reducing caloric intake and body weight in rodents with diet-induced or genetic obesity than in their respective lean controls (Di Marzo et al., 2001b; Hildebrandt et al., 2003; Ravinet Trillouet et al., 2003; Vickers et al., 2003).

Second, $CB_1^{-/-}$ mice are resistant to diet-induced obesity (Ravinet Trillou et al., 2004; Osei-Hyiaman et al., 2005b). In both of these studies, overall caloric intake was not different between wild-type compared with $CB_1^{-/-}$ mice receiving the high-fat diet, suggesting that peripheral mechanisms play a dominant role in the control of body weight by CB_1 receptors. $CB_1^{-/-}$ mice are also resistant to the metabolic changes that accompany diet-induced obesity in normal mice, including hypertriglyceridemia and elevated plasma leptin and insulin levels, indicative of leptin and insulin resistance, respectively (Ravinet Trillou et al., 2004; Osei-Hyiaman et al., 2005b). These metabolic changes, collectively defined by some as the "metabolic syndrome", could also be reversed by SR141716 treatment (Ravinet Trillou et al., 2004; Poirier et al., 2005).

As a third line of evidence, recent findings indicate that endocannabinoids and CB_1 receptors are up-regulated in the liver and adipose tissue in various forms of experimental as well as in human obesity. In wild-type mice on a high-fat diet for 3 weeks, the basal rate of de novo hepatic fatty acid synthesis was markedly in-

creased, and the increase was partially reversed by SR141716 treatment (Osei-Hyiaman et al., 2005b). After 3 weeks of diet, the mice were not yet overweight but showed significant hepatic steatosis. Their hepatic content of anandamide was increased 3-fold, and the level of CB_1 receptor protein in liver plasma membranes was also markedly increased (Osei-Hyiaman et al., 2005b). These findings indicate that intake of a high-fat diet activates the hepatic endocannabinoid system, which contributes to increased lipogenesis and the subsequent development of hepatic steatosis and, ultimately, the development of obesity. Exposure of C57BL6/J mice to a high-fat diet has been reported to induce changes characteristic of the metabolic syndrome and also to rapidly induce the expression of SREBP1c and its downstream target lipogenic enzymes (Biddinger et al., 2005). CB_1 receptor knockout mice are resistant to these diet-induced changes, which indicates that endocannabinoids have a major role in mediating them (Osei-Hyiaman et al., 2005b).

An up-regulation of CB_1 receptors has been also reported in adipose tissue of genetically obese compared with lean mice (Bensaid et al., 2003), and elevated endocannabinoid levels have been detected in adipose tissue of obese compared with lean patients (Matias et al., 2006). In a study involving 40 women (Engeli et al., 2005), circulating levels of anandamide and 2-AG were significantly increased in 20 obese versus 20 lean subjects, and remained elevated after a 5% diet-induced weight reduction. Although these plasma levels were much too low to exert hormone-like activity, they probably originate from overflow from tissues and thus may reflect functionally relevant changes in endocannabinoid content at or near sites of action. In the same study, FAAH expression was markedly reduced in the adipose tissue of obese subjects and correlated negatively with circulating endocannabinoid levels. Furthermore, the expression of both CB_1 and FAAH increased in mature adipocytes compared with preadipocytes. These findings suggest that the endocannabinoid system is activated in human obesity (Engeli et al., 2005).

A genetic missense polymorphism in the *FAAH* gene predicting a proline to threonine substitution at position 129, which was reported to result in reduced cellular expression and activity of the enzyme (Chiang et al., 2004), had been earlier found to be significantly associated with problem drug use (Sipe et al., 2002). The same polymorphism has been linked to overweight and obesity in both Caucasian and African-American subjects (Sipe et al., 2005). Interestingly, the elevated hepatic levels of anandamide in mice receiving a high-fat diet could be attributed to a decrease in FAAH activity (Osei-Hyiaman et al., 2005b), suggesting that FAAH may play a key role in regulating endocannabinoid "tone" in both experimental and human obesity. Although this finding could suggest the targeting of FAAH in the treatment of eating/metabolic disorders, such an approach will be complicated by the fact that oleylethanolamide, an anorectic lipid that acts on the peroxisome proliferator-proliferator-activated receptor α (PPARα) (Fu et al., 2003), is also a substrate for FAAH. The opposing effects of elevated levels of both anandamide and oleylethanolamide after pharmacological blockade of FAAH may therefore result in no net change in appetite and energy metabolism.

That increased endocannabinoid activity may also contribute to obesity and its metabolic consequences in humans was indicated by the highly promising results of recent clinical trials with rimonabant. As in the animal models of diet-induced obesity, rimonabant was effective both in reducing body weight and in reversing many of the associated metabolic abnormalities in obese subjects. In a multicenter, phase III study involving 1507 obese European subjects with a body mass index >30 kg/m^2 or a body mass index >27 kg/m^2 with dyslipidemia and moderate hypertension, rimonabant (20 mg/day) treatment for 1 year, combined with a moderately hypocaloric diet, not only reduced body weight but also reduced plasma triglycerides, increased HDL cholesterol, and decreased plasma insulin and insulin resistance (Van Gaal et al., 2005). Blood pressure was not significantly affected. The parallel reduction in body weight and waist circumference suggested that the weight loss was predominantly due to loss of visceral fat, which is known to be a predisposing factor for the metabolic syndrome. Rimonabant was well tolerated, with mild to moderate nausea, diarrhea, and mood disorders occurring slightly more in the treatment group than in the placebo group (Van Gaal et al., 2005).

Essentially similar findings were reported in another large-scale, phase III study (RIO-North America) involving 3045 randomized, obese or overweight subjects. At the end of the 1st year, rimonabant-treated subjects were re-randomized to receive rimonabant or placebo, whereas the placebo group continued onto receive the placebo. During the 2nd year, rimonabant-treated patients retained the improvements achieved during the 1st year, whereas those who switched to placebo regained their original weight (Pi-Sunyer et al., 2006).

In a third study (RIO-Lipids) involving 1036 overweight/obese subjects, 20 mg/day rimonabant taken for 1 year significantly reduced body weight (-6.3 ± 0.5 kg), weight circumference (-5.7 ± 0.6 cm), and plasma triglycerides ($-12.4 \pm 3.2\%$), increased HDL cholesterol by $8.1 \pm 1.5\%$ and increased LDL particle size, improved glucose tolerance, and significantly elevated plasma adiponectin levels, resulting in a 50% decrease in the prevalence of the metabolic syndrome in the study population (Després et al., 2005). In contrast with the other two studies, a statistically significant, small decrease in systolic and diastolic blood pressure was evident in the group receiving 20 mg of rimonabant, and the decrease was greater for patients with initial hypertension (blood pressure >140/90 mm Hg). Although the reason for the

lack of a blood pressure change in the other studies is not clear, the proportion of females was lower in RIO-Lipids (~60%) than in the other two studies where they represented ~80% of subjects. It is possible that a modest reduction in blood pressure by rimonabant occurs preferentially in males. The cumulative finding that blood pressure reduction, if present, is less than expected based on a similar level of weight reduction alone (Appel et al., 2003), is noteworthy. As discussed in section D.1., rimonabant at an i.v. dose of 3 mg/kg causes a pressor response in anesthetized, hypertensive rats, which are supersensitive to the hypotensive effect of endogenous or exogenous anandamide (Bátkai et al., 2004). Although the pressor effect is much smaller at lower doses of rimonabant comparable with the 20-mg oral dose used in humans or in the absence of anesthesia (S. Bátkai, P. Pacher, and G. Kunos, unpublished observations), careful monitoring of blood pressure, particularly in the early stages of rimonabant treatment, may be advisable. A polymorphism in the *FAAH* gene is associated with obesity (Sipe et al., 2005), and because of the reduced enzyme activity resulting from this polymorphism, some of the affected individuals may have an elevated endocannabinoid tone, reversal of which by rimonabant could increase blood pressure.

It is noteworthy that part of the rimonabant-induced improvements in the hormonal and lipid abnormalities in the three clinical studies appeared to be independent of weight reduction and, based on the preclinical findings discussed above, are most likely mediated via peripheral sites of action. An interesting alternative mechanism is suggested by the results of a recent meta-analysis of the effects of low carbohydrate, nonenergy-restricted diets on weight loss and cardiovascular risk factors (Nordmann et al., 2006). Such diets were found to lead to significant weight loss for up to 1 year. Surprisingly, they were more favorable than low-fat diets in reducing plasma triglycerides and increasing HDL cholesterol levels, without a favorable effect on total or LDL cholesterol. The pattern of these metabolic changes is similar to that of those caused by 20 mg of rimonabant in the three clinical trials. Rimonabant has been shown to preferentially suppress the preference for sweet compared with normal (Simiand et al., 1998) or high-fat reinforcers (Ward and Dykstra, 2005) and can cause longer lasting suppression of intake of sweet compared with normal food (Gessa et al., 2006). It is very possible that obese subjects treated with rimonabant unwittingly altered their diet by reducing carbohydrate intake, which may have contributed to the observed effects on triglycerides and HDL cholesterol. Detailed analyses of the effects of rimonabant on dietary habits are warranted.

Overall, the findings in these three large, multicenter clinical trials strongly support a pathogenic role of increased endocannabinoid activity in obesity and the associated metabolic abnormalities and highlight the unique therapeutic potential of CB_1 blockade. Additional benefits may be gained by combination therapies. The efficacy of statins to preferentially lower LDL cholesterol may be effectively complemented by the ability of rimonabant to increase HDL cholesterol. In the case of insulin, the ability of rimonabant ability to increase insulin sensitivity could reduce the dose requirement for insulin in obese diabetic subjects and could also counteract the tendency of insulin treatment to cause weight gain. Nevertheless, further large-scale studies are warranted in view of the high nonadherence rate observed in the three clinical trials to date, which may have resulted in overestimation of the benefits of treatment (Simons-Morton et al., 2006).

4. Cachexia and Anorexia. A negative energy balance resulting from decreased appetite and food intake and increased energy expenditure, leading to weight loss, can be the consequence of wasting diseases such as AIDS or metastatic cancer, or it could be associated with aging, chemotherapy of cancer, or neuropsychiatric conditions such as anorexia nervosa or various forms of dementia including Alzheimer's disease. Although there is a growing body of evidence documenting the therapeutic effectiveness of synthetic THC or even smoked marijuana as appetite boosters in some of these conditions (Regelson et al., 1976; Gorter et al., 1992; Nelson et al., 1994; Beal et al., 1995, 1997; Timpone et al., 1997) (Table 1), there is only limited information on the potential involvement of the endocannabinoid system in their pathogenesis.

A few studies have reported the effectiveness of THC in stimulating appetite and weight gain in cancer patients, but these therapeutic effects have been more extensively documented in AIDS patients (reviewed by Kirkham, 2004; Martin and Wiley, 2004; Hall et al., 2005) (see also Table 1). Although concerns have been voiced about the potential immunosuppressive effect of cannabinoids in immunocompromised individuals (Klein et al., 1998), repeated THC administration in a randomized, prospective, controlled trial was found to have few if any consistent effects on various immune functions in AIDS patients receiving antiviral treatment (Bredt et al., 2002).

Anorexia may also be associated with normal aging. A number of hormonal factors have been implicated in the loss of appetite in the elderly, including growth hormone, cholecystokinin, leptin, and various cytokines (Morley, 2001). In a recent study in mice, an age-related decline in food and alcohol intake was accompanied by the loss of ability of the CB_1 antagonist SR141716 to reduce food and alcohol intake and a decrease in CB_1 receptor-stimulated GTPγS labeling in the limbic forebrain (Wang et al., 2003). These findings suggest that, at least in this animal model, an age-dependent decrease in CB_1 receptor signaling in the limbic forebrain may be related to the parallel decline in appetite for both food and alcohol. Anorexia can also accompany debilitating

diseases such as Alzheimer's disease, in which the effectiveness of THC to stimulate appetite has been documented (Volicer et al., 1997). Anorexia nervosa is a psychiatric condition that occurs predominantly in younger women and is characterized by self-starvation, weight loss, and a disturbed body image. Plasma anandamide levels have been reported to increase in patients with restricting anorexia nervosa, which may be secondary to a marked decrease in plasma leptin levels in such patients (Monteleone et al., 2002). Although the relationship between brain and plasma levels of anandamide is not clear, a parallel increase in anandamide in brain regions involved in reward may mediate the rewarding effect of self-starvation in anorexic patients (Monteleone et al., 2005). A recent family-based study examined the possible association of a CB_1 receptor gene polymorphism consisting of differences in a trinucleotide repeat with anorexia nervosa. Although no difference was found between parental alleles transmitted or not transmitted to the affected siblings, preferential transmission of different alleles could be established when the patients were subdivided into restricting and binging/purging subgroups (Siegfried et al., 2004).

Endocannabinoids have been also implicated in a unique form of food intake: milk suckling in newborn animals. In an elegant series of studies, Fride et al. (2005) have proposed a role for 2-AG in the brain to stimulate the suckling response in mouse pups. In their model, endogenous 2-AG in the pup's brain initiated the suckling response via CB_1 receptors, with continued suckling depending on milk-derived 2-AG (Fride, 2004). As predicted by this model, treatment of pups with SR141716 inhibits suckling and leads to death due to failure to thrive, a condition analogous to a human condition known as nonorganic failure to thrive, in which an oral motor defect resulting in deficient suckling (Reilly et al., 1999) is similar to the condition in mouse produced by pharmacological blockade or genetic ablation of CB_1 (Fride et al., 2005). The relatively high dose of SR141716 to inhibit suckling and its residual effectiveness in CB_1 knockout mice suggested the additional involvement of a receptor distinct from CB_1 or CB_2 (Fride et al., 2003).

B. Pain and Inflammation

One of the earliest uses of cannabis was to treat pain. Historical documents reveal the use of cannabis for surgical anesthesia in ancient China and to relieve pain of diverse origin in ancient Israel, Greece, Rome, and India (reviewed in Mechoulam, 1986; Iversen, 2000; Mechoulam and Hanus, 2000). Numerous early studies have also demonstrated beneficial effects of cannabinoids in animal models of pain (reviewed in Walker and Huang, 2002; Fox and Bevan, 2005). In acute pain, anandamide, THC, cannabidiol, and synthetic cannabinoids such as CP55,940 and WIN 55,212-2 are effective against chemical (Sofia et al., 1973; Formukong et al., 1988; Calignano et al., 1998; Costa et al., 2004a,b; Guindon et al., 2006; Ulugol et al., 2006), mechanical (Sofia et al., 1973; Martin et al., 1996; Smith et al., 1998; Guindon and Beaulieu, 2006), and thermal pain stimuli (Buxbaum, 1972; Bloom et al., 1977; Lichtman and Martin, 1991a,b; Fride and Mechoulam, 1993; Guindon and Beaulieu, 2006). Recent animal studies indicate that anandamide and cannabinoid ligands are also very effective against chronic pain of both neuropathic (Herzberg et al., 1997; Bridges et al., 2001; Fox et al., 2001; Guindon and Beaulieu, 2006) and inflammatory origin (Tsou et al., 1996; Richardson et al., 1998a,b,c; Li et al., 1999; Martin et al., 1999b; Guindon et al., 2006). Moreover, endocannabinoids and synthetic cannabinoids exert synergistic antinociceptive effects when combined with commonly used nonsteroid anti-inflammatory drugs, which may have utility in the pharmacotherapy of pain (Guindon and Beaulieu, 2006; Guindon et al., 2006; Ulugol et al., 2006). Interestingly, a recent study has implicated the endocannabinoid system in the analgesic activity of paracetamol (acetaminophen), the most widely used painkiller (Ottani et al., 2006), and there is also evidence for endocannabinoid involvement in the action of some general anesthetics, such as propofol (Patel et al., 2003; Schelling et al., 2006).

Cannabinoids exert their antinociceptive effects by complex mechanisms involving effects on the central nervous system (Martin et al., 1993; Hohmann et al., 1995, 1998, 1999; Martin et al., 1995, 1996, 1998, 1999a,b; Richardson et al., 1997, 1998a,b; Meng et al., 1998; Strangman et al., 1998; Hohmann and Walker, 1999; Fox et al., 2001), spinal cord (Yaksh, 1981; Lichtman and Martin, 1991a,b; Welch and Stevens, 1992; Richardson et al., 1997, 1998a,b; Hohmann et al., 1998; Chapman, 1999; Drew et al., 2000; Naderi et al., 2005; Suplita et al., 2006), and peripheral sensory nerves (Calignano et al., 1998; Richardson et al., 1998c; Hohmann and Herkenham, 1999; Fox et al., 2001; Johanek et al., 2001; Kelly et al., 2003; Johanek and Simone, 2004; Jordt et al., 2004; Amaya et al., 2006). This is consistent with the anatomical location of CB_1 receptors in areas relevant to pain in the brain, spinal dorsal horn, dorsal root ganglia, and peripheral afferent neurons (Herkenham et al., 1990, 1991a; Hohmann and Herkenham, 1998, 1999; Hohmann et al., 1999; Sañudo-Peña et al., 1999a).

In addition to the role of CB_1 receptors, there is recent evidence implicating CB_2 receptors in the antihyperalgesic activity of cannabinoids in models of acute and chronic, neuropathic pain, especially of inflammatory origin (Calignano et al., 1998; Hanus et al., 1999; Malan et al., 2001; Clayton et al., 2002; Ibrahim et al., 2003, 2005; Nackley et al., 2003a,b, 2004; Quartilho et al., 2003; Elmes et al., 2004; Hohmann et al., 2004; Scott et al., 2004; Whiteside et al., 2005; Ibrahim et al., 2006). Cannabinoid agonists may also release endogenous opioids, and a functional interplay between the endocan-

nabinoid and opioid systems in modulating analgesic responses has been suggested by numerous studies (Pugh et al., 1997; Manzanares et al., 1999a,b; Houser et al., 2000; Ibrahim et al., 2005; Tham et al., 2005; Vigano et al., 2005a,b; Williams et al., 2006).

As discussed before, anandamide is also a ligand for $TRPV_1$ receptors, albeit with an affinity lower than its affinity for CB_1 receptors. The potential involvement of $TRPV_1$ in the analgesic effect of endogenous anandamide has been raised by the findings that the analgesic response to microinjection of a FAAH antagonist into the periaqueductal gray of rats could be inhibited by a similar local microinjection of 6 nmol of capsazepine (Maione et al., 2006). However, others reported that systemic administration of 10 mg/kg capsazepine, which blocked capsaicin-induced analgesia, failed to inhibit endocannabinoid-mediated, stress-induced analgesia, which could be enhanced by a FAAH inhibitor and completely blocked by the CB_1 antagonist rimonabant (Suplita et al., 2006).

The analgesic response to exogenous cannabinoids suggested a role for the endocannabinoid system in regulating pain sensitivity, which has received experimental support (reviewed in Walker et al., 2000, 2002; Cravatt et al., 2004; Boger et al., 2005). For example, Walker et al. (1999) have demonstrated increased anandamide levels in some brain areas involved in nociception after peripheral nociceptive input in the rat. The functional role of endogenous anandamide was further supported by the predominantly CB_1-mediated analgesic response to FAAH or endocannabinoid transport inhibitors in animal models of acute and chronic pain (Lichtman et al., 2004a; Chang et al., 2006; Jayamanne et al., 2006; La Rana et al., 2006; Suplita et al., 2006). Similarly, FAAH knockout mice had elevated brain levels of anandamide and displayed analgesic behavior in acute inflammatory, but not in chronic neuropathic models of pain (Lichtman et al., 2004b). Formation of anandamide and 2-AG is also increased in response to stress in the periaqueductal gray matter, in which inhibition of endocannabinoid degradation was found to enhance stress-induced analgesia in a CB_1 receptor-dependent manner (Hohmann et al., 2005; Suplita et al., 2006), confirming and extending an earlier finding that implicated CB_1 receptors and endocannabinoids in stress-induced analgesia (Valverde et al., 2000).

In humans, the analgesic activity of THC and other cannabinoids is less clear-cut. There are numerous case reports on the beneficial effects of cannabis or synthetic derivatives of THC in pain associated with multiple sclerosis, cancer, neuropathies, and HIV infection (Noyes et al., 1975a,b; Martyn et al., 1995; Consroe et al., 1997; Hamann and di Vadi, 1999; Ware et al., 2003; Rudich et al., 2003; Ware and Beaulieu, 2005; Ware et al., 2005; Berlach et al., 2006; reviewed in Burns and Ineck, 2006) (Table 1). The results of randomized studies conducted before 1999 on the analgesic effect of orally administered synthetic cannabinoids in patients with postoperative, post-traumatic, cancer, or spastic pain had been subjected to a meta-analysis. The authors concluded that cannabinoids were not more effective than codeine in controlling pain, and their use was associated with numerous undesirable, dose-limiting CNS side effects (Campbell et al., 2001).

Recent clinical trials with THC or cannabis extracts containing a 1:1 mixture of THC and cannabidiol (Sativex, GW-1000) have provided mixed results. In a randomized, double-blind, placebo-controlled crossover study of 48 patients suffering from central neuropathic pain due to brachial plexus avulsion, oromucosally administered THC or Sativex was ineffective in reducing the pain severity score recorded during the last 7 days of treatment (Berman et al., 2004). Similarly, oral THC (dronabinol) did not improve postoperative (Buggy et al., 2003) and neuropathic pain (Attal et al., 2004) in trials involving small numbers of patients. However, numerous lessons have been learned from these initial human studies on optimal trial design, dose and route of administration of cannabinoids, and more recent larger-scale studies allow reason for more optimism, as outlined below.

THC or Sativex reduced neuropathic pain in patients with traumatic nerve injury or multiple sclerosis in randomized, double-blind, placebo-controlled, crossover trials (Wade et al., 2003; Notcutt et al., 2004). Modest, but clinically relevant analgesic effects were reported in 21 multiple sclerosis patients treated with dronabinol, in a randomized, controlled clinical trial (Svendsen et al., 2004). Effective pain relief by orally administered cannabis extract or THC was also reported in a randomized, controlled, multicenter trial involving 611 multiple sclerosis patients (Zajicek et al., 2003). Moreover, in a recent study of 66 multiple sclerosis patients, Sativex was effective in reducing central neuropathic pain (Rog et al., 2005). A preview of as-yet-unpublished human studies gave an account of a significant benefit of Sativex over placebo in peripheral neuropathic pain characterized by allodynia, in central pain associated with multiple sclerosis, and in opiate-resistant, intractable pain due to cancer (Russo, 2006). A multicenter dose-escalation study of the analgesic and adverse effects of an oral cannabis extract (Cannador) in patients with postoperative pain demonstrated significant dose-related improvements in rescue analgesia requirements and significant trends across the escalating dose groups for decreasing pain intensity (Holdcroft et al. 2006). THC (Marinol) was found to suppress otherwise intractable cholestatic pruritus in a case report (Neff et al., 2002). An analysis of pain questionnaires from 523 patients with HIV infections revealed that 90 to 94% of the subjects using cannabis experienced improvement in muscle and neuropathic pain (Woolridge et al., 2005). The therapeutic potential of cannabinoids in pain associated with trigeminal neuralgia and migraine has also been the

subject of several recent reviews (Liang et al., 2004; Russo, 2004, 2006). Preclinical studies (Burstein et al., 1998, 2004; Burstein, 2000, 2005; Dyson et al., 2005; Mitchell et al., 2005; Salim et al., 2005) and a recent clinical trial of 24 patients with neuropathic pain of varying etiologies demonstrated that ajulemic acid, a major metabolite of THC with CB_1 agonist activity, was effective in reducing pain without causing cannabinoid-like CNS side effects, the first evidence for the separability of the psychotropic and analgesic effects of a THC analog in humans (Karst et al., 2003). Numerous additional human studies are ongoing to determine the effectiveness of THC or cannabis-based extracts against various forms of pain (reviewed in Ware and Beaulieu, 2005, 2006).

Multiple lines of evidence support the important role of the cannabinoid signaling system in the modulation of immune function and inflammation (reviewed in Klein et al., 1998, 2003; Walter and Stella, 2004; Klein, 2005). First, cannabinoid receptors are present on immune cells, where their expression is modulated by microbial antigens or other stimuli that induce immune activation. Second, stimulation of immune cells by bacterial toxins such as lipopolysaccharide (LPS) increases the cellular levels of endocannabinoids and their degrading enzyme(s). Third, cannabinoid agonists modulate immune function both in vitro and in vivo via cannabinoid receptor-dependent and -independent mechanisms.

The anti-inflammatory effects of cannabinoids are complex and may involve modulation of cytokine (e.g., TNF-α, IL-12, IL-1, IL-6, and IL-10) and chemokine production (e.g., CCL2, CCL5, CXCL8, and CXCL10), modulation of adenosine signaling (Carrier et al., 2006), expression of adhesion molecules (e.g., ICAM-1, P- intercellular adhesion molecule-1 and P-selectin), and the migration, proliferation, and apoptosis of inflammatory cells (reviewed in Klein et al., 1998, 2003; Walter and Stella, 2004; Klein, 2005). To the extent that pain and inflammation accompany many of the disorders discussed in the rest of this review, cannabinoids would be expected to provide significant benefit due to their analgesic and anti-inflammatory properties.

C. Central Nervous System Disorders

The emerging role of the endocannabinoid system in a variety of CNS disorders should not come as a surprise given the very high level of expression of CB_1 receptors in the brain. The particularly high density of CB_1 receptors in the cortex, cerebellum, hippocampus, and basal ganglia had drawn early attention to diseases affecting movement, mood and anxiety disorders, and conditions related to altered brain reward mechanisms, as well as processes of memory and learning. The classic behavioral effects of marijuana also provided early clues about potential therapeutic targets, such as the control of pain or appetite. The role of the endocannabinoid system in the pathogenesis and treatment of specific CNS diseases is discussed below.

1. Neurotoxicity and Neurotrauma. The endocannabinoid system plays an important role in neuroprotection both in acute neuronal injury (e.g., traumatic brain injury, stroke, and epilepsy) and in chronic neurodegenerative disorders, such as multiple sclerosis, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Alzheimer's disease (reviewed in Glass, 2001; Mechoulam et al., 2002a,b; Grundy, 2002; Croxford, 2003; Drysdale and Platt, 2003; Jackson et al., 2005a; Ramos et al., 2005). Although the underlying mechanisms are not fully understood, multiple cannabinoid receptor-dependent as well as receptor-independent processes have been implicated. These include, but are not limited to 1) modulation of excitatory glutamatergic transmissions and synaptic plasticity via presynaptic CB_1 receptors (Molina-Holgado et al., 1997; Marsicano and Lutz, 1999; Gerdeman et al., 2002; reviewed in Alger, 2002; Robbe et al., 2002; Azad et al., 2003; Freund et al., 2003; Gerdeman and Lovinger, 2003; Piomelli, 2003; Mato et al., 2004), 2) modulation of immune responses and the release of inflammatory mediators by CB_1, CB_2, and non CB_1/CB_2 receptors on neurons, astrocytes, microglia, macrophages, neutrophils and lymphocytes (Watzl et al., 1991; Zheng et al., 1992; Fischer-Stenger et al., 1993; Cabral and Fischer-Stenger, 1994; Kusher et al., 1994; Burnette-Curley and Cabral, 1995; Cabral et al., 1995; reviewed in Friedman et al., 1995; Zheng and Specter, 1996; Shohami et al., 1997; Newton et al., 1998; Srivastava et al., 1998; Gallily et al., 2000; Klein et al., 2000a,b, 2003; Smith et al., 2000; Carlisle et al., 2002; Germain et al., 2002; Killestein et al., 2003; Kaplan et al., 2005; Ramirez et al., 2005; reviewed in Friedman et al., 1995; Stella, 2004; Walter and Stella, 2004; Correa et al., 2005; Croxford and Yamamura, 2005; Klein, 2005;), 3) activation of cytoprotective signaling pathways (Grigorenko et al., 2002), such as protein kinase B/Akt (Molina-Holgado et al., 2002), protein kinase A (Kim et al., 2005), or neurotrophic factors (Khaspekov et al., 2004), 4) modulation of excitability and calcium homeostasis via effects on Ca^{2+}, K^+, and Na^+ channels, *N*-methyl D-aspartate (NMDA) receptors, gap junctions, and intracellular Ca^{2+} stores (Caulfield and Brown, 1992; Mackie and Hille, 1992; Mackie et al., 1993; Nadler et al., 1995; Venance et al., 1995; Shohami et al., 1997; Hampson et al., 2000b; Oz et al., 2000, 2004; Chemin et al., 2001; Maingret et al., 2001; Nogueron et al., 2001; Robbe et al., 2001; Wilson and Nicoll, 2001; Wilson et al., 2001; Nicholson et al., 2003; Guo and Ikeda, 2004; del Carmen et al., 2005; del Carmen Godino et al., 2005; Zhuang et al., 2005), 5) antioxidant properties of cannabinoids (Eshhar et al., 1995; Hampson et al., 1998; Chen and Buck, 2000; reviewed in Hampson et al., 2000a; Marsicano et al., 2002a), and 6) CB_1 receptor-mediated hypothermia, possibly by reducing metabolic rate and oxygen demand (Leker et al., 2003).

Excitotoxicity, the toxic effects of an overactivation of glutamate receptors, and the resulting oxidative stress may contribute to the pathological processes eventually leading to cellular dysfunction or death in both acute and chronic forms of neurodegeneration (Coyle and Puttfarcken, 1993; McNamara, 1999; Lutz, 2004). Dexanabinol (HU-211), a behaviorally inactive cannabinoid and noncompetitive antagonist of NMDA receptors, protects primary rat neuronal cultures against NMDA and glutamate exposure in vitro (Eshhar et al., 1993; Nadler et al., 1993a,b). THC protects primary cultured neurons against kainate-mediated toxicity in a CB_1-dependent manner (Abood et al., 2001), similar to protectin by WIN 55,212-2 against low extracellular magnesium-induced cell death (Shen and Thayer, 1998). Palmitoylethanolamide also improves neuronal survival in a glutamate-induced cell death model (Skaper et al., 1996). Intracerebral injection of NMDA in neonatal rats results in a 13-fold increase of cortical anandamide concentrations (Hansen et al., 2001a,b). Both THC and anandamide exerted CB_1-mediated neuroprotective effects in an ouabain-induced rat model of in vivo excitotoxicity (van de Stelt et al., 2001a,b). Anandamide and synthetic agonists of CB_1 receptors also protected the newborn brain against AMPA-kainate receptor-mediated excitotoxic damage in mice (Shouman et al., 2006).

Traumatic brain injury (TBI) is one of the leading causes of disability and mortality in young individuals (Holm et al., 2005), yet the available therapy is very limited (Faden, 2002; Maas et al., 2004). TBI is characterized by cerebral edema, axonal and neuronal injury, increased permeability of the blood-brain barrier, and post-traumatic changes in cognitive and neurological functions (Bayir et al., 2003). TBI can trigger glutamate-induced excitotoxicity, oxidative stress, release of inflammatory cytokines from brain-resident cells (microglia, neurons, and astrocytes), programmed cell death, and cortical blood flow dysregulation (reviewed in Wang and Feuerstein, 2000; Gentleman et al., 2004).

The protective effect of cannabinoids in traumatic brain injury was first indicated in studies with the non-psychotropic cannabinoid dexanabinol (HU-211) (Fig. 1b). These studies have demonstrated reduced brain damage and improved motor and cognitive function in HU-211-treated animals in a rat model of TBI. The favorable effects of a single injection of HU-211 on learning and neurological deficits lasted up to 30 days and could be achieved within a therapeutic window of 6 h (Shohami et al., 1993, 1995). Beneficial effects of HU-211 were also demonstrated in an axonal injury model (Yoles et al., 1996; Zalish and Lavie, 2003). These protective effects were attributed, at least in part, to NMDA receptor blockade, attenuation of Ca^{2+} influx and decreased TNF-α levels (Nadler et al., 1995; Shohami et al., 1997; reviewed in Mechoulam et al., 2002a,b; Biegon, 2004). In mice with closed head injury, brain levels of 2-AG increased, and exogenous 2-AG administered 1 h after the head injury reduced infarct size and improved neurological outcome (Panikashvili et al., 2001). Neuroprotection by 2-AG was attributed to CB_1 receptor-mediated inhibition of nuclear factor-κB and of the early expression of proinflammatory cytokines TNF-α, IL-1β, and IL-6 (Panikashvili et al., 2005, 2006). In a rat model of TBI, BAY 38-7271, a CB_1/CB_2 agonist with predominant action at CB_1 receptors, caused a marked, 70% reduction in infarct volume when administered as a 4-h infusion immediately after induction of subdural hematoma, and even when it was applied with a 3-h delay, infarct volume was reduced by 59% (Mauler et al., 2002).

A multicenter, double-blind, randomized, placebo-controlled phase II trial conducted in 67 patients with severe closed head injury found dexanabinol to be safe and well tolerated. The treated patients achieved significantly better intracranial pressure/cerebral perfusion pressure control without jeopardizing blood pressure, and a trend toward faster improvement and better neurological outcome was also observed (Knoller et al., 2002). However, a double-blind, randomized, placebo-controlled phase III clinical trial of dexanabinol, conducted in 15 countries in 86 specialized centers and involving 861 patients failed to demonstrate any favorable effects (Maas et al., 2006).

2. Stroke. Ischemic stroke is the most common form of stroke, mostly caused by a transient interruption of blood supply to the brain by thrombotic occlusion of blood vessels. It is an important cause of death and disability in industrialized countries, affecting up to 0.2% of the population each year (Klijn and Hankey, 2003; Pinto et al., 2004). One in six patients die in the 1st month after ischemic stroke, and half of the survivors are permanently disabled despite the best efforts to rehabilitate them and to prevent complications (Klijn and Hankey, 2003).

One of the first indications of the neuroprotective effect of cannabinoids came from the field of stroke research, using various in vitro and in vivo models of ischemic injury. Anandamide, 2-AG, and WIN 55,212-2 protected cultured cortical neurons against hypoxia and glucose deprivation (Nagayama et al., 1999; Sinor et al., 2000). The effects of various cannabinoid ligands were also investigated in in vivo models of global cerebral ischemia induced by two-vessel occlusion with hypotension or by four-vessel occlusion, or in focal ischemia induced by occlusion of the middle cerebral artery (MCAo), with or without reperfusion. Dexanabinol at doses of 2 to 10 mg/kg decreased infarct size and histological damage and improved neurological score in rat and gerbil models of both global and focal cerebral ischemia (Bar-Joseph et al., 1994; Vered et al., 1994a,b; Belayev et al., 1995a,b,c; Leker et al., 1999; Lavie et al., 2001; Teichner et al., 2003). Importantly, this protective effect was observed even when the drug was administered 60 to 180 min after the insult (Vered et al., 1994;

Belayev et al., 1995a,b,c; Leker et al., 1999; Lavie et al., 2001; Teichner et al., 2003).

WIN 55,212-2, at doses of 0.03 and 1 mg/kg but not 3 mg/kg decreased hippocampal neuronal loss after transient global cerebral ischemia in rats. It also reduced infarct size after permanent focal cerebral ischemia induced by MCAo, when given 40 min before 30 min after the occlusion, and these effects were prevented by SR141716 (Nagayama et al., 1999). WIN 55,212-2 also protected cultured cerebral cortical neurons from in vitro hypoxia and glucose deprivation, but in contrast to the receptor-mediated neuroprotection observed in vivo, this in vitro effect was not stereoselective and was insensitive to CB_1 and CB_2 receptor antagonists (Nagayama et al., 1999). BAY38-7271 also decreased infarct size in rats with permanent MCAo even when given intravenously 4 h after the occlusion (Mauler et al., 2002). Similarly, HU-210 reduced infarct size by up to 77% and improved motor disability in a rat model of permanent MCAo (Leker et al., 2003). The protective effect of HU-210 was partially reversed by pretreatment with SR141716, indicating CB_1 receptor involvement. Surprisingly, all protection could be abolished by warming the animals to the body temperature of controls, indicating that CB_1-mediated hypothermia contributed to the neuroprotection (Leker et al., 2003). Likewise, CB_1-mediated hypothermia was responsible for the neuroprotective effects of THC in a mouse transient MCAo model (Hayakawa et al., 2004) and perhaps also in a rat model of global cerebral ischemia (Louw et al., 2000). Consistent with these findings, CB_1 knockout mice had increased mortality from permanent focal cerebral ischemia, increased infarct size, more severe neurological deficits after transient focal cerebral ischemia, and decreased cerebral blood flow in the ischemic penumbra during reperfusion, compared with wild type controls subjected to the same insult (Parmentier-Batteur et al., 2002). NMDA neurotoxicity was also increased in $CB_1^{-/-}$ mice compared with wild-type littermates (Parmentier-Batteur et al., 2002). Further evidence for a role of CB_1 receptors is their increased expression on neurons in the arterial boundary zone of cortical infarction (Jin et al., 2000). Finally, brain levels of endocannabinoids are increased during ischemic (Schmid et al., 1995; Schabitz et al., 2002; Berger et al., 2004; Muthian et al., 2004) and other types of brain injury (Sugiura et al., 2000; Hansen et al., 2001a,b; Panikashvili et al., 2001).

Other studies do not support the neuroprotective role of CB_1 receptor activation. For example, CB_1 antagonists by themselves had no effect on the outcome of injury, and in two recent reports, SR141716 and LY320135 were found to actually reduce infarct size and to improve neurological function in a rat model of MCAo (Berger et al., 2004; Muthian et al., 2004), whereas low doses of WIN 55,212-2 had no protective effect (Muthian et al., 2004). Thus, it appears that both CB_1 agonists and antagonists can be neuroprotective in cerebral ischemia. The reason for the opposite effects of pharmacological blockade versus genetic knockout of CB_1 receptors is not clear and may be related to the CB_1 receptor-independent effects of antagonists (Begg et al., 2005; Pertwee, 2005b,c). Clearly, evaluating the potential usefulness of cannabinoid ligands in the treatment of stroke warrants future studies.

3. Multiple Sclerosis and Spinal Cord Injury. Multiple sclerosis (MS) is a complex, immune-mediated, inflammatory disease of the white matter of the brain, which compromises impulse conduction due to the loss of the myelin sheath of neurons and the secondary axonal loss (Sospedra and Martin, 2005). MS affects 2 to 5 million people worldwide and commonly presents with an unpredictable, relapsing-remitting course and a range of clinical symptoms depending on where the demyelination and axonal loss have occurred (Compston and Coles, 2002). Some patients become disabled within a short period of time, whereas others can live their entire lives with only negligible or no disability. The symptoms of MS typically involve tremor, ataxia, visual loss, double vision, weakness or paralysis, difficulty in speaking, loss of bladder control and constipation, cognitive impairment, and painful muscle spasms. Muscle spasticity often leads to reduced mobility, considerable distress from pain, and interference with daily living activities. Spasticity, neuropathic and nociceptive pain, and some of the above symptoms are also common in spinal cord injury (SCI). Although there are numerous drugs available that target the immune system to slow down the progression of the disease, they are only moderately effective, and the treatment of MS remains mostly symptomatic and far from satisfactory (Killestein and Polman, 2005).

Cannabis had been used in ancient Greece, Rome, China, and India for relieving muscle cramps, spasm, and pain (reviewed in Mechoulam, 1986, Mechoulam et al., 1998; Mechoulam and Hanus, 2000) and its therapeutic application in MS is a topic of recent lively debate (Grundy, 2002; Pertwee, 2002; Baker and Pryce, 2003; Croxford, 2003; Killestein et al., 2004; Sirven and Berg, 2004; Jackson et al., 2005a; Pryce and Baker, 2005; Robson, 2005; Smith, 2005). Lyman et al. (1989) examined the effects of parenteral THC in experimental autoimmune encephalomyelitis (EAE) in rats, a laboratory model of MS. THC treatment not only reduced CNS inflammation and improved neurological outcome but also improved survival compared with placebo. Δ^8-THC, a less psychotropic and more stable analog of THC, also reduced the severity and incidence of neurological deficits in rats with EAE (Wirguin et al., 1994). The nonpsychotropic dexanabinol also suppressed inflammatory responses in the brain and spinal cord of rats with EAE and improved their neurological symptoms (Achiron et al., 2000). Although CB_1 receptor density is decreased in the striatum and cortex of EAE rats, this is compensated for by increased coupling to G protein-mediated signal-

ing, ensuring the effectiveness of treatment with cannabinoid agonists (Berrendero et al., 2001).

In a mouse model of chronic relapsing EAE, intravenous administration of THC, WIN 55,212-2, JWH-133, or methanandamide reduced spasticity and tremor, whereas the same symptoms were exacerbated by treatment with either CB_1 or CB_2 antagonists (Baker et al., 2000). These mice with EAE had increased levels of anandamide, 2-AG, and palmitoylethanolamide (PEA) in areas associated with nerve damage (Baker et al., 2001). Furthermore, spasticity could be relieved not only by administration of exogenous anandamide, 2-AG, or PEA but also by selective inhibitors of endocannabinoid transport or hydrolysis, which suggests tonic control of muscle tone by the endocannabinoid system in EAE (Baker et al., 2001; Ligresti et al., 2006a). Additional evidence for this has emerged through the use of CB_1-deficient mice, which tolerated inflammatory and excitotoxic insults poorly and developed substantial neurodegeneration after the induction of EAE (Pryce et al., 2003). Jackson et al. (2005b) reported that the absence of CB_1 receptors was associated with increased caspase activation and a greater loss of myelin and axonal/neuronal proteins after the induction of chronic EAE. Interestingly, CB_1 knockout mice had increased caspase 3 levels before the induction of EAE, suggesting a neuroprotective tone mediated by CB_1 receptors (Jackson et al., 2005a,b). In mice with EAE, WIN 55,212-2 inhibited leukocyte/endothelial interactions via activation of CB_2 receptors (Ni et al., 2004). Interestingly, a recent study suggests that the high levels of IFN-γ present in the CNS of mice with EAE can counteract endocannabinoid-mediated neuroprotection by disrupting P2X7 purinergic receptor signaling, a key step in endocannabinoid production by glia (Witting et al., 2006).

Another murine model of MS is Theiler's murine encephalomyelitis virus-induced demyelinating disease. In mice with Theiler's murine encephalomyelitis virus-induced demyelinating disease, treatment with WIN 55,212-2 slowed the progression of symptoms, down-regulated delayed-type hypersensitivity reactions and interferon-γ production, and inhibited the expression of proinflammatory cytokines in the CNS (Croxford and Miller, 2003). In another study using this model, treatment with WIN 55,212-2, ACEA, or JWH-015 caused long-lasting improvements in neurological deficits in the established disease and reduced microglial activation, abrogated major histocompatibility complex class II antigen expression, and decreased the number of $CD4^+$ infiltrating T cells in the spinal cord. These changes were paralleled by extensive remyelination (Arevalo-Martin et al., 2003). Treatment of Theiler's murine encephalomyelitis virus-infected mice with the transport inhibitors OMDM1 and OMDM2 enhanced anandamide levels, down-regulated inflammatory responses in the spinal cord, and ameliorated motor symptoms (Mestre et al., 2005), and similar findings were reported using the transport inhibitor UCM707 (Ortega-Gutierrez et al., 2005). In these two studies, the treatments were also shown to reduce the surface expression of major histocompatibility complex class II molecules, the production of the proinflammatory cytokines (TNFα, IL-1β, and IL-6), and the expression of inducible NO synthase.

Consistent with the animal data, cannabinoids have shown promise in the treatment of MS in humans (Table 1). A possible underlying mechanism is suggested by a recent study in which the endocannabinoid system was found to be highly activated during CNS inflammation in MS patients and to protect neurons from inflammatory damage by activating a negative feedback loop in microglial cells via $CB_{1/2}$-mediated epigenetic regulation of mitogen-activated protein kinase phosphatase 1 expression (Eljaschewitsch et al., 2006).

There have been anecdotal reports of the effectiveness of marijuana smoking in relieving symptoms of MS and SPI (Grinspoon and Bakalar, 1993, 1998), which were supported by the results of early open or single-blind observations with orally given THC or smoked marijuana, involving small numbers of patients (Dunn and Davis, 1974; Petro, 1980; Petro and Ellenberger, 1981; Clifford, 1983; Meinck et al., 1989; Brenneisen et al., 1996; Schon et al., 1999). The most consistent finding was a subjective improvement in spasticity, although benefits for mobility, tremor, nystagmus, mood, and bladder control were also reported. In a double-blind crossover study of a single MS patient, nabilone treatment improved muscle spasms, nocturia, and general well-being (Martyn, 1995). In contrast, Greenberg et al. (1994) reported impairments of both balance and posture after a single dose of smoked cannabis in a placebo-controlled study of 10 MS patients and 10 normal subjects. In an anonymous survey of 112 MS patients who self-medicated with cannabis, 30 to 97% of the subjects reported relief from a wide variety of symptoms by smoking marijuana (Consroe et al., 1997). These encouraging reports have triggered numerous larger, population-based clinical trials of cannabis-based medicines in MS, which have yielded mixed results.

Using a randomized, double-blind, placebo-controlled, crossover design, Killestein et al. (2002) have evaluated the effects of oral THC, two doses of 2.5 to 5 mg/day or a *Cannabis sativa* plant extract administered over a 4-week period, in 16 MS patients with severe spasticity. Spasticity and disability, quantified using the objective Ashworth scale (Ashworth, 1964) and the Expanded Disability Status Scale were not improved. However, a significant improvement in the subjective rating of spasm frequency and trends toward improved mobility were noted, with no effect on tremor, sleep quality, or lower urinary tract symptoms. Both THC and the plant extract worsened the patients' global impression of their conditions, with plant extracts causing more adverse side effects. It should be mentioned, however, that the dose of THC used was lower than that in subsequent

studies with more positive outcome, and as was noted in an accompanying editorial (Thompson and Baker, 2002), the study was not powered to detect efficacy.

A large multicenter study involving 33 clinical centers and 660 MS patients in the United Kingdom and United States and supported by the UK Medical Council aimed to explore the effects of cannabis extract (Cannador) or synthetic THC (Marinol) versus placebo on spasticity, pain, tremor, bladder function, and cognitive function [Cannabinoids in Multiple Sclerosis (CAMS) study; Zajicek et al., 2003, 2004]. There was no change in Ashworth score, tremor, irritability, depression, or tiredness after 15 weeks of treatment with Marinol or Cannador. However, there were significant improvements in patient-reported spasticity, pain, and sleep quality. Unexpectedly, there was also a reduction in hospital admissions for relapse in the two active treatment groups. Adverse side effects were generally minor and similar to those with placebo. Remarkably, in the 12-month follow-up of the original CAMS study of 657 patients, muscle spasticity measured by the Ashworth scale was significantly improved in the THC-treated group. The Rivermead Mobility Index was also improved, indicative of reduced disability. The effect of Cannador on tremor was also studied in a randomized, double-blind, placebo-controlled, crossover trial in 14 patients with MS. Consistent with an earlier report (Zajicek et al., 2003), no significant therapeutic effects were noted (Fox et al., 2004). In another study of similar design, administration of oral capsules containing 2.5 mg of Δ^9-THC plus 0.9 mg of CBD (maximal dose of 30 mg of Δ^9-THC/day) caused improvements in spasm frequency and mobility in 37 MS patients who received at least 90% of their prescribed dose (Vaney et al., 2004).

In a double-blind, placebo-controlled study involving 18 patients with MS, THC and CBD decreased self-reported spasticity and pain and improved bladder symptoms, whereas spasticity measured by the Ashworth scale was not significantly improved (Wade et al., 2003). The therapeutic effect of Sativex delivered by oromucosal spray (2.7 mg of THC and 2.5 mg of CBD at each actuation) was evaluated in 160 outpatients with MS (Smith, 2004). Patients were allowed to self-titrate the dose to achieve optimal effects, up to a maximal daily dose of 120 mg of THC and CBD. Efficacy was assessed by using a modified Ashworth scale to assess spasticity, whereas daily living, mobility, cognitive function, and tremor were quantified through the use of various scales and questionnaires (Wade et al., 2004). There was no significant difference in the Ashworth scale, tremor, and pain at 6 weeks between the Sativex and placebo groups. However, visual analog scales showed significant improvement in patients whose primary symptom had been spasticity (Wade et al., 2004). Sativex was well tolerated and effective against central neuropathic pain and sleep disturbances associated with MS in a randomized, controlled trial involving 66 patients (Rog et al., 2005). Sativex was approved and launched in Canada in 2005 for the treatment of neuropathic pain associated with MS and is currently being investigated for various other therapeutic indications (Russo, 2004, 2006).

In a recent case report, a 46-year-old woman was diagnosed with MS after having entered treatment with the CB_1 receptor antagonist rimonabant for obesity, and recovery to near normal was noted within weeks after discontinuation of the treatment (van Oosten et al., 2004). This report, coupled with the more severe neurodegenerative process when MS is induced in CB_1 knockout mice or in mice treated with a CB_1 receptor antagonist, could suggest that CB_1 antagonism may exacerbate inflammatory demyelinating diseases in humans (van Oosten et al., 2004). However, the occurrence of MS in this one patient may have been purely coincidental.

Although the results of the above clinical studies (Table 1) are somewhat equivocal, patients treated with cannabis experienced improvements in the most disturbing symptoms including pain and spasticity compared with those receiving placebo, without experiencing significant side effects. These studies also suggest that the Ashworth scale as a primary measure of spasticity in MS does not accurately assess the complex collection of symptoms associated with spasticity, which may be more accurately evaluated using subjective measures. Indeed, the use of the Ashworth scale as a primary measure of spasticity in MS has often being criticized, and many commonly used antispasticity medications have also failed to generate statistically significant improvements according to this scale (Hinderer and Gupta, 1996; Shakespeare et al., 2003). Accurate assessment of the clinical effectiveness of cannabinoids in MS may be complicated by the difficulty in achieving the most appropriate individual oral dose (Table 1). Peak plasma concentrations and their timing vary greatly because of the low water solubility of cannabis components and the large variability in their absorption from the gastrointestinal tract. An additional disadvantage of oral administration is the hepatic first-pass effect. This can result in the formation of large quantities of the psychoactive metabolite 11-OH-THC, which may be responsible for some of the side effects observed (Table 1). Delivery of cannabis-based extracts as an oromucosal spray may minimize these drawbacks and may allow patients to better optimize their individual daily dose by self-titration (Russo, 2006).

In conclusion, controlled clinical trials with cannabinoids have demonstrated their efficacy in eliciting symptomatic improvements in MS patients. These results suggest that there is place for the use of cannabis in the treatment of MS, which should be confirmed in further larger-scale clinical trials.

4. Movement Disorders (Basal Ganglia Disorders). Endocannabinoid involvement in the central regulation of motor functions and in movement disorders is

based on multiple lines of evidence. First, CB_1 receptors are highly expressed in the basal ganglia, especially in the substantia nigra and in the cerebellum (Herkenham et al., 1990, 1991a,b; Mailleux and Vanderhaeghen, 1992; Tsou et al., 1998; Hohmann and Herkenham, 2000; Moldrich and Wenger, 2000; Howlett et al., 2002), areas involved in motor control. Second, endocannabinoids are also abundant in these brain regions (Bisogno et al., 1999a; Di Marzo et al., 2000). Third, endogenous, plant-derived, and synthetic cannabinoids have potent, mostly inhibitory, effects on motor activity (Crawley et al., 1993; Fride and Mechoulam, 1993; Wickens and Pertwee, 1993; Smith et al., 1994; Romero et al., 1995a,b, 2002b; reviewed in Sañudo-Peña et al., 1999b). Fourth, CB_1 receptor and endocannabinoid levels are altered in the basal ganglia both in experimental models (Zeng et al., 1999; Page et al., 2000; Romero et al., 2000; Lastres-Becker et al., 2001a,b, 2002a,b; Gonzalez et al., 2006) and human forms of movement disorders (Glass et al., 1993, 2000, 2004; Lastres-Becker et al., 2001a; reviewed in Romero et al., 2002b). Fifth, the endocannabinoid system interacts with several neurotransmitter pathways at various levels of the basal ganglia circuitry (Glass et al., 1997a; Miller et al., 1998; Sañudo-Peña and Walker, 1998; Giuffrida et al., 1999; Rodriguez De Fonseca et al., 2001; Brotchie, 2003; van der Stelt and Di Marzo, 2003; de Lago et al., 2004a).

a. Parkinson's disease and levodopa-induced dyskinesia. Parkinson's disease (PD) is the second most common neurodegenerative disease of adult onset, with incidence of 16 to 19/100,000 people worldwide (Twelves et al., 2003). PD is caused by a severe loss of dopaminergic neurons in the substantia nigra pars reticulata (SNr), resulting in reduced dopamine levels and a loss of dopaminergic neurotransmission in the striatum, which interferes with motor function and coordination. Although excitotoxicity, oxidative stress, inflammation, mitochondrial dysfunction, and environmental and hereditary factors have all been implicated in the pathogenesis of PD, the exact cause of the loss of dopaminergic neurons remains elusive (Hattori and Mizuno, 2004; Eriksen et al., 2005). Clinically, PD is characterized by the classic triad of resting tremor, muscular rigidity, and bradykinesia/akinesia (slowness of movement or postural immobility). Current therapies include oral dopamine replacement via the dopamine precursor levodopa, anticholinergic agents, and monoamine oxidase B inhibitors (Horn and Stern, 2004). Although dopamine replacement therapy can be effective in most patients by controlling the symptoms in the short term, their long-term use is associated with diminishing efficacy and severe side effects such as levodopa-induced dyskinesia (LID) (involuntary movements), which often lead to treatment discontinuation and severe disability.

In PD, there are secondary abnormalities in nondopaminergic transmission within the basal ganglia that are thought to contribute to the inhibition of motor function. Inhibitory GABAergic transmission from the striatum to the external region of the globus pallidus (GPe) is increased, making the GPe hypoactive. This results in decreased GABAergic input from the GPe to the subthalamic nucleus which, together with increased activity of glutamatergic efferents to this nucleus, results in its hyperactivity. In turn, the hyperactive subthalamic nucleus increases the activity of the SNr and internal globus pallidus (GPi) through glutamatergic efferents. Because both the SNr and GPi provide inhibitory output to motor nuclei outside the basal ganglia (e.g., motor thalamus and brain stem locomotor regions), this mechanism is thought to contribute to the excessive motor inhibition in PD (Obeso et al., 2000; Bezard et al., 2001). In general, changes opposite to those described above are likely to be involved in LID. The final outcomes of the dysregulation of neuronal circuits are abnormal patterning, firing rate, and synchronization of basal ganglia outputs (Obeso et al., 2000; Bezard et al., 2001). Importantly, nondopaminergic mechanisms may counterbalance the loss of dopamine and are probably responsible for the lack of parkinsonian symptoms until the loss of >80% of striatal dopamine. They may also attenuate the severity of symptoms once symptoms develop. As discussed below, the endocannabinoid system may play an important regulatory role in PD,PD and LID as well as in the compensatory mechanisms.

Overactivity of endocannabinoid transmission, as reflected by increased tissue levels of endocannabinoids and CB_1 receptors as well as decreased rates of endocannabinoid transport and degradation by FAAH, have been found in the basal ganglia in the 6-hydroxydopamine-lesioned or reserpine-treated rat models of PD (Mailleux and Vanderhaeghen, 1993; Romero et al., 2000; Gubellini et al., 2002; Centonze et al., 2005; Fernandez-Espejo et al., 2005; Gonzalez et al., 2006). In basal ganglia from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned marmosets, a primate model of PD, and in basal ganglia of PD patients, the density of striatal CB_1 receptors and CB_1 receptor-G-protein coupling were found to be increased (Lastres-Becker et al., 2001a). The above changes in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated marmosets and 6-hydroxydopamine-lesioned rats were reversible by chronic L-dopa treatment, which indicates that the similar changes observed in PD patients were unlikely to have been induced by the replacement therapy (Lastres-Becker et al., 2001a; Maccarrone et al., 2003). There is broad agreement that the endocannabinoid system becomes overactive in the basal ganglia in PD (reviewed in Brotchie, 2003), although some studies report a reduction (Silverdale et al., 2001) or no change in CB_1 receptor expression (Herkenham et al., 1991a) or a dependence on L-DOPA cotreatment of the increased CB_1 receptor expression in the basal ganglia of animals with experimental PD (Zeng et al., 1999).

If the enhanced CB_1 receptor signaling in the striatum is viewed as an attempt of the dopamine-deficient brain to normalize striatal function, the pharmacological amplification of this signaling might alleviate symptoms of PD, e.g., by reducing striatal glutamate release (Gerdeman and Lovinger, 2001; Gerdeman et al., 2002; Gubellini et al., 2002). On the other hand, enhanced CB_1 receptor signaling, if focused on the other part of the circuitry (e.g., GPe), can enhance GABA transmission, leading to inhibition of GPe and thereby contributing to the symptoms of PD. Likewise, CB_1 antagonism could have either pro-parkinsonian effects, if it targets the striatum, or antiparkinsonian effects, if it targets the GPe. Accordingly, both agonists and antagonists might have therapeutic potential, both in PD and LID (reviewed in Brotchie, 2003).

Treatment with CB_1 receptor agonists can decrease the tremor associated with overactivity of the subthalamic nucleus (Sañudo-Peña et al., 1998, 1999b), improve motor impairment seen with dopaminergic agonists (Anderson et al., 1995; Maneuf et al., 1997; Sañudo-Peña et al., 1998), protect against dopaminergic cell death (Lastres-Becker et al., 2005), and delay or reduce the incidence of LID (Sieradzan et al., 2001; Fox et al., 2002a; Ferrer et al., 2003; Gilgun-Sherkiet et al., 2003). However, cannabinoid agonists are unlikely to be used for reducing bradykinesia in PD because of their hypokinetic profile both in primates and humans (Consroe, 1998; Müller-Vahl et al., 1999a; Romero et al., 2002; Brotchie, 2003; Croxford, 2003; Croxford and Miller, 2003).

On the other hand, dysfunction of nigrostriatal dopaminergic neurons can be associated with overactivity of endocannabinoid transmission in the basal ganglia (see above). CB_1 receptor antagonists may therefore be useful for alleviating the bradykinesia of PD or LID, because they attenuate CB_1 signaling in GPe or GPi. (Mailleux and Vanderhaeghen, 1993; Di Marzo et al., 2000; Lastres-Becker et al., 2001a,b; Gubellini et al., 2002; reviewed in Brotchie, 2003; Fernandez-Espejo et al., reviewed in Brotchie, 2003; 2005; Fernandez-Ruiz and Gonzalez, 2005). Notwithstanding the above, studies using SR141716 in rat (Di Marzo et al., 2000) and primate models of PD or LID (Meschler et al., 2001; van der Stelt et al., 2005) provided conflicting results. Rimonabant treatment also failed to influence dyskinesia in the first small-scale, randomized, double-blind, placebo-controlled human study (Mesnage et al., 2004). However, the dose used in this human study was approximately 10-fold lower (0.3 mg/kg versus. 3 mg/kg), than in a recent primate study with positive outcome (van der Stelt et al., 2005). As suggested by a recent report (Fernandez-Espejo et al., 2005), it is also possible that CB_1 receptor blockade is effective only at the very advanced stages of the disease. More recently, using Park-2 knockout mice, a genetic model of early PD, Gonzalez et al. (2005) observed gender-dependent differences for both the levels of CB_1 receptors and motor responses to agonists or antagonists, extending earlier data obtained in humans and in animal models of PD.

Taken together, although the above studies do not offer a complete understanding of the role of endocannabinoids and cannabinoid receptors in PD and LID, they support the notion that the endocannabinoid system plays an important role in movement disorders, including PD, and may provide the framework for novel therapeutic approaches in the future.

b. Huntington's disease. Huntington's disease (HD) is an inherited, autosomal dominant, progressive neuropsychiatric disorder of the midlife, caused by an unstable expansion of a trinucleotide polyglutamine repeat in the N-terminal domain of a protein termed huntingtin, which leads to degeneration of neurons in the basal ganglia and cortical regions. The disease is characterized by motor disturbances, such as chorea (involuntary movements) and dystonia, psychiatric symptoms, and dementia (Melone et al., 2005). The prevalence of HD is similar to that of ALS (see below), but much lower than that of most of the other neurodegenerative illnesses discussed above or below. The therapy of HD is very limited and includes antidopaminergic drugs to reduce the hyperkinesias and antiglutamatergic agents to reduce excitotoxicity (Melone et al., 2005).

It has been clearly demonstrated, both in postmortem human tissue (Glass et al., 1993, 2000; Richfield and Herkenham, 1994) and in chemically induced and transgenic animal models (Denovan-Wright and Robertson, 2000; Page et al., 2000; Lastres-Becker et al., 2001, 2002a,b; Sieradzan and Mann, 2001; Behrens et al., 2002; Glass et al., 2004; McCaw et al., 2004) that a decrease in CB_1 receptor level and signaling activity in the basal ganglia is one of the earliest changes in HD, preceding nerve loss and clinical symptoms. Furthermore, decreased levels of anandamide and 2-AG in the striatum and an increase of anandamide in the ventral mesencephalon, where the substantia nigra is located, have been documented in a rat model of HD (Lastres-Becker et al., 2001). Thus, it appears that endocannabinoid signaling in the basal ganglia is hypofunctional in HD, which probably contributes to the hyperkinesia associated with the disease. These studies also suggest that the endocannabinoid system is involved in the pathogenesis and/or progression of HD, and cannabinoid agonists could be of significant therapeutic benefit in HD because of their anthyperkinetic and neuroprotective effects (reviewed in Lastres-Becker et al., 2003b). A recent study identified a novel population of progenitor cells expressing CB_1 receptors in the subependymal layer of the normal and Huntington's diseased human brain. This finding raises the intriguing possibility that these cells could be a source of replacement of cells lost due to neurodegenerative disease (Curtis et al., 2006).

Indeed, data from animal models demonstrated that both CB_1 agonists and inhibitors of endocannabinoid

transport are able to reduce hyperkinesia (Lastres-Becker et al., 2002b, 2003a). Interestingly, direct agonists of CB_1 receptors, such as CP55,940, only produced a very modest effect compared with the anandamide transport inhibitor AM404, which also exhibits affinity for the VR_1 receptor (Zygmunt et al., 2000). This latter property of AM404 may account for its ability to reduce hyperkinesia (Lastres-Becker et al., 2002b, 2003a), as other transport inhibitors such as VDM11 and AM374, which are not active at $TRPV_1$ receptors, were devoid of antihyperkinetic effects in HD rats (Lastres-Becker et al., 2003a), and the most potent transport inhibitor to date, UCM707, only produced a modest effect (de Lago et al., 2002, 2004b, 2006). Arvanil, a hybrid endocannabinoid and vanilloid compound, was also reported to alleviate hyperkinesias in a rat model of HD (de Lago et al., 2005). These results suggest that $TRPV_1$ receptors alone, or in combination with CB_1 receptors, might represent novel therapeutic targets in HD (reviewed in Lastres-Becker et al., 2003b).

There have been few human trials on the effects of cannabinoid agonists in HD, and the results do not live up to the promise of the animal data. Small trials with the synthetic THC analog nabilone and with the nonpsychoactive cannabidiol showed no efficacy or even increased choreic movements in HD patients (Consroe et al., 1991; Müller-Vahl et al., 1999b). These negative results could be related to dosing issues, to the lack of $TRPV_1$ receptor activity of the compounds tested, or to the advanced stage of the disease. Nevertheless, further studies are warranted to explore the therapeutic potential of cannabinoids in HD.

c. Gilles de la Tourette's syndrome, tardive dyskinesia, and dystonia. Based on its ubiquitous presence in motor regions of the brain, the endocannabinoid system might be involved in other extrapyramidal disorders such as Gilles de la Tourette's syndrome (TS), tardive dyskinesia, and dystonia. TS is a neurological syndrome that becomes evident in early childhood and is characterized by multiple motor and vocal tics lasting for more than 1 year. Plant-derived cannabinoids have been found to be effective in the treatment of tics and behavioral problems in TS (Müller-Vahl et al., 1997, 1998, 1999c, 2002, 2003a,b; Müller-Vahl, 2003). Beneficial effects of cannabinoids have been also reported in dystonia, both in animal models (Richter and Löscher, 1994, 2002) and in humans (Fox et al., 2002b; Jabusch et al., 2004). In addition, as described in the sections above, cannabinoids have potential in the management of the LID in PD and of the spasticity and tremor in MS. On the other hand, in patients chronically treated with neuroleptic drugs, a correlation between chronic cannabis use and the presence of tardive dyskinesia has been described previously (Zaretsky et al., 1993).

5. Amyotrophic Lateral Sclerosis. ALS (also known as Lou Gehrig's disease) is the most common adult-onset human motor neuron disease with a prevalence of 5 to 7/100,000. It is characterized by rapid, progressive degeneration of motor neurons in the brain and spinal cord, which ultimately leads to progressive weakness, paralysis, and premature death (Rowland and Shneider, 2001). Although weak, patients are cognitively intact and thus are completely aware of their progressive disability. The disease strikes adults at any age, and most patients die within 3 to 5 years after the onset of symptoms. Although most cases of ALS are sporadic and are probably acquired, approximately 10% are familial, usually inherited in an autosomal dominant pattern. Despite a variety of putative underlying mechanisms, including oxidative stress, neuroinflammation, autoimmunity, a defect in neuronal glutamate transport and glutamate toxicity, neurofilament accumulation, exogenous factors (virusesor toxins), mitochondrial dysfunction, and mutations in the superoxide dismutase (*SOD1*) gene, the pathogenesis of ALS is incompletely understood (Barnham et al., 2004). Tragically, available treatment options are limited and do not prevent disease progression and death (Rowland and Shneider, 2001).

Based on the well-known protective effect of cannabinoids against oxidative cell damage and excitotoxicity (Hampson et al., 1998; Shen and Thayer, 1999; Abood et al., 2001; van der Stelt et al., 2001a), combined with their antispastic effect in MS, Carter and Rosen (2001) have proposed the use of marijuana for the pharmacological management of ALS. Indeed, in a pilot study of the safety and tolerability of THC in ALS patients, symptomatic benefits were seen for spasticity, insomnia, and appetite (Gelinas et al., 2002). Consistent with this clinical report, studies using transgenic mice expressing a mutant form of human SOD1 ($hSOD1^{G93A}$ mice) as an experimental model of ALS have demonstrated that either THC or WIN55,212-2 administered after the onset of the disease or genetic ablation of FAAH delayed disease progression (Raman et al., 2004; Bilsland et al., 2006). Furthermore, THC potently reduced oxidative and excitotoxic damage in spinal cord cultures in vitro and prolonged survival in SOD1 mutant mice (Raman et al., 2004). Surprisingly, neither WIN55,212-2 nor FAAH ablation affected the life span of SOD1(G93A) mice, whereas deletion of the CB_1 receptor significantly extended life span without affecting the disease onset (Bilsland et al., 2006). These results suggest that cannabinoids have significant neuroprotective effects in a mouse model of ALS but that these beneficial effects may be mediated by non-CB_1 receptor mechanisms.

6. Alzheimer's Disease. Alzheimer's disease (AD) is a progressive neurodegenerative disorder that accounts for the vast majority of age-related dementia and is one of the most serious health problems in the industrialized world. The disease is characterized by the formation of

neuritic plaques rich in β-amyloid (Aβ) peptide, neurofibrillary tangles rich in hyperphosphorylated τ protein, gliosis, and a neuroinflammatory response involving astrocytes and microglia, inevitably leading to progressive global cognitive decline (Weksler et al., 2005). These studies have engendered new perspectives on the possible role of the endocannabinoid system in neurodegenerative processes associated with inflammation (reviewed in Walter and Stella, 2004), including those in AD (reviewed in Pazos et al., 2004).

In an in vitro cell culture model of AD, anandamide prevented Aβ-induced neurotoxicity through CB_1-mediated activation of the mitogen-activated protein kinase pathway (Milton, 2002). In rat microglia cells in culture, CB_1 receptor stimulation also dose dependently inhibited the release of NO, which had been implicated in the neurotoxic effects of Aβ peptide (Waksman et al., 1999). In PC12 cells, protection against Aβ-induced neurotoxicity was also observed with cannabidiol, which does not bind to CB_1/CB_2 receptors (Iuvone et al., 2004). Interestingly, CB_1 receptor blockade by SR141716 improved the memory deficit induced by administration of Aβ peptide in mice, presumably by increasing hippocampal acetylcholine levels (Mazzola et al., 2003). However, analyses of brain tissue samples obtained from AD patients (Westlake et al., 1994) or animal models of AD (Romero et al., 1998; Benito et al., 2003) indicate that CB_1 receptors are not dramatically affected. In contrast, CB_2 receptors and FAAH are overexpressed in microglia associated with neuritic plaques in the brain of AD patients (Benito et al., 2003). Senile plaques in AD patients express both CB_1 and CB_2 receptors together with markers of microglial activation, and CB_1-positive neurons, present in high numbers in control cases, are greatly reduced in areas of microglial activation (Ramirez et al., 2005). CB_1 receptor protein levels and G protein coupling were also markedly decreased in AD brains, coupled with increased nitration of the CB_1 and CB_2 receptor proteins (Ramirez et al., 2005). Intracerebroventricular administration of WIN 55,212-2 to rats prevented Aβ-induced microglial activation, cognitive impairment and loss of neuronal markers. HU-210, WIN 55,212-2, and JWH-133 blocked Aβ-induced activation of cultured microglial cells, as judged by mitochondrial activity, cell morphology and TNF-α release, and these effects were independent of the antioxidant action of ligands. Furthermore, cannabinoids abrogated microglia-mediated neurotoxicity after addition of Aβ to rat cortical cocultures (Ramirez et al., 2005). Although there are no data available on the endocannabinoid content in AD brain tissue are available, increased levels have been reported in the brain after inflammatory events and in neurodegenerative disorders associated with inflammation (reviewed in Walter and Stella, 2004 and see also sections above).

Based on the above, one might hypothesize that Aβ deposition induces the release of endocannabinoids from neurons and glia, which activate CB_1-mediated neuroprotective pathways and modulate the release of inflammatory mediators in microglia through CB_2 receptors. If this hypothesis is confirmed by future studies, the beneficial effects of CB_1/CB_2 agonists and FAAH antagonists in AD could be explored. Intriguingly, in a recent open-label pilot study of six patients in the late stages of dementia (five patients with AD and one patient with vascular dementia), treatment with 2.5 mg of dronabinol daily for 2 weeks significantly improved the Neuropsychiatric Inventory total score and the subscores for agitation and aberrant motor and nighttime behaviors (Walther et al., 2006).

7. Epilepsy. If the balance between inhibitory and excitatory communications among neurons is disturbed, the intensity of excitatory transmission may exceed a certain threshold, leading to epileptic seizures. Stimulation of postsynaptic neurons is known to trigger the on-demand synthesis of endocannabinoids via an increase in intracellular calcium and/or stimulation of metabotropic receptors (reviewed in Piomelli, 2003; Lutz, 2004). Thereafter, endocannabinoids are released and reach presynaptic CB_1 receptors retrogradely to modulate both inhibitory GABAergic and excitatory glutamatergic transmissions via multiple mechanisms (Marsicano and Lutz, 1999; Alger, 2002, 2004; Gerdeman et al., 2002; Robbe et al., 2002; Azad et al., 2003; Freund et al., 2003; Gerdeman and Lovinger, 2003; Kim and Alger, 2004; Isokawa and Alger, 2005).

Multiple pathways, eventually culminating in neuronal death, are triggered by excessive excitatory activity through a process known as excitotoxicity (McNamara, 1999). Excitotoxicity is believed to contribute to the progression of numerous degenerative central nervous system disorders such as Parkinson's disease, Alzheimer's disease, and various forms of epilepsy. More than 1% of the human population is affected by epilepsy and the incidence is highest in elderly persons or during the first years of life (reviewed in Holmes and Ben-Ari, 1998; McCormick and Contreras, 2001). Epileptic syndromes are classified as generalized seizures, which affect the entire forebrain, or partial seizures, which occur within localized brain regions. Conventional antiepileptic treatment is not fully effective in ~30% of patients, therefore justifying the search for new targets (LaRoche and Helmers, 2004).

Cannabis has been used to treat epilepsy for centuries. Hashish was reported to cure the sick son of the chamberlain of the Caliphate Council in Baghdad by the medieval Arab writer Ibn al-Badri (Mechoulam, 1986; Iversen, 2000). Almost four centuries later, W. B. O'Shaughnessy, an Irish physician and scientist working at the Medical College of Calcutta, confirmed the benefit of hashish for treating pain, emesis, mus-

cle spasms, and convulsions (reviewed in Karler and Turkanis, 1981; Mechoulam, 1986). The benefit of cannabis in epilepsy was also reported by a British neurologist (Reynolds, 1890), but the medicinal use of cannabis was prohibited in the early 20th century in most countries.

After the identification of the structure of THC (Gaoni and Mechoulam, 1964), several groups investigated its antiepileptic effects (reviewed in Gordon and Devinsky, 2001; Lutz, 2004). THC was originally characterized as an anticonvulsant, but it has a variety of excitatory and depressant effects, ranging from convulsions to ataxia, depending on the dose, experimental model, and the animal species used (Karler and Turkanis, 1981; reviewed in Gordon and Devinsky, 2001; Lutz, 2004). Further complicating the picture, animal studies also document a rebound effect to THC with enhanced CNS excitability and increased sensitivity to convulsions (Chiu et al., 1979; Karler and Turkanis, 1981; Karler et al., 1986). This withdrawal hypersensitivity implies that in susceptible patients, the use of marijuana may be associated with withdrawal seizures (Karler and Turkanis, 1981).

Only case reports on the effects of THC in epileptic patients are currently available. Two reports described decreased seizure frequency after marijuana use (Consroe et al., 1975; Ellison et al., 1990) and an epidemiological study found that chronic marijuana use is protective against seizures (Ng et al., 1990). According to a questionnaire completed by 215 epileptic patients using marijuana regularly, 7.4% experienced a reduction, 2.3% an increase, and 90.2% no change in seizure frequency (Gordon and Devinsky, 2001). In contrast, marijuana smoking was associated with an increase in seizure frequency in another study (Keeler and Reifler, 1967). Small-scale clinical studies have shown that the nonpsychotropic cannabidiol either reduced seizure frequency or had no significant effect on it (Cunha et al., 1980; Ames and Cridland, 1986; Gordon and Devinsky, 2001).

As in human studies, cannabinoids were found to exert both anti- and proconvulsive activity in animal models of epilepsy, largely depending on the stimulus applied to induce seizures (chemical, electrical, light, or fever) and the species used (Johnson et al., 1975; Ten Ham et al., 1975; Wada et al., 1975a,b; Corcoran et al., 1978; Chiu et al., 1979; Duncan and Dagirmanjian, 1979; Fish et al., 1981; Karler and Turkanis, 1981; Colasanti et al., 1982; Fish and Consroe, 1983; Karler et al., 1984, 1986; Consroe and Mechoulam, 1987; Pertwee et al., 1991; Hayase et al., 2001a,b; reviewed in Gordon and Devinsky, 2001; Lutz, 2004).

Anandamide and its metabolically stable analog, O-1812, dose dependently inhibited electroshock-induced seizures in rats, and this effect was abolished by SR141716 (Wallace et al., 2002). In a rat model of febrile seizures, the expression of presynaptic CB_1 receptors in hippocampal GABAergic interneurons was increased (Chen et al., 2003), and the CB_1 receptor-mediated DSI was enhanced (Alger, 2002), suggesting that the endogenous cannabinoid system is protective. Remarkably, in a rat model of pilocarpine-induced status epilepticus, CB_1 receptor agonists were more effective in reducing seizure frequency than clinically used anticonvulsants, such as phenytoin or phenobarbital. Consistently, CB_1 receptor blockade increased seizure frequency, and the seizure activity was associated with increased brain levels of CB_1 receptors and 2-AG (Wallace et al., 2003a).

With use of the kainic acid-induced excitotoxic epileptiform seizure model in wild type and CB_1 knockout mice, recent studies have established that the seizure-induced increase of intracellular calcium, a hallmark of epilepsy (Raza et al., 2001), triggers the synthesis of anandamide, which activates CB_1 receptors in glutamatergic neurons in the hippocampus and cerebral cortex (Marsicano et al., 2003; Khaspekov et al., 2004). Such "on-demand" activation of CB_1 receptors was suggested to protect against excitotoxicity by various mechanisms, including inhibition of calcium channels and stimulation of potassium channels to decrease neuronal excitability and the activation of extracellular signal regulated kinases (Marsicano et al., 2003; Khaspekov et al., 2004). In contrast to these findings, FAAH knockout mice or mice treated with a CB_1 agonist were found to have increased sensitivity to kainic acid-induced seizures (Clement et al., 2003). The lack of protection in this latter study may be related to the nonselective activation of CB_1 receptors on both inhibitory (proconvulsive effect) and excitatory neurons (anticonvulsive effect) and by the life-long rather than on-demand activation of CB_1 receptors present in FAAH knockout animals.

In summary, the use of cannabinoids for the treatment of epilepsy is still controversial, although recent experimental studies provide some new insight. To date, there have been no large-scale, controlled clinical trials to examine the beneficial effects of cannabinoids in various forms of epilepsy. The potential use of the nonpsychotropic cannabidiol and of inhibitors of anandamide transport or degradation warrants further investigation.

8. Mental Disorders. The well-known psychotropic effects of cannabinoids and the distribution of cannabinoid receptors across important emotional circuits in the brain suggest that the endocannabinoid system may be involved in various psychiatric disorders such as schizophrenia and mood disorders (reviewed in van der Stelt and Di Marzo, 2003; Hall et al., 2004; Leweke et al., 2004; Manzanares et al., 2004; Ujike and Morita, 2004; Ashton et al., 2005; Gambi et al., 2005; Semple et al., 2005; Vinod and Hungund, 2005).

a. Schizophrenia. Schizophrenia is the second most common mental disorder with a lifetime prevalence of approximately 0.2 to 2% worldwide (Ban, 2004). The disease usually begins in early adulthood or late adoles-

cence and is characterized by psychotic episodes with positive symptoms including delusions and/or hallucinations, loose associations, and distortion of perception. The psychotic episodes are separated by periods with negative symptoms consisting of apathy, anhedonia, reduced social drive, loss of motivation, poverty of speech and thought, and blunting of affect. With disease progression, behavioral impairment can lead to complete social isolation. Although recent advances in the pharmacotherapy of schizophrenia produced great improvement in the clinical symptoms and the quality of life of patients, there is room for further improvements (Ban et al., 2004; Moller, 2005).

Numerous theories have been put forth regarding the etiology of schizophrenia, ranging from developmental or neurodegenerative processes, environmental factors, neurotransmitter abnormalities (dopamine or glutamate), and infectious or autoimmune processes, but also including the cannabinoid hypothesis (reviewed in Thaker and Carpenter, 2001; Lewis et al., 2005). It appears that hypoglutamatergic and hypodopaminergic transmission in the prefrontal cortex is involved in the negative symptoms, whereas hyperactivity of dopamine neurotransmission in the mesencephalic projections to the nucleus accumbens may underlie the positive symptoms (Thaker and Carpenter, 2001; Lewis et al., 2005).

According to the endocannabinoid hypothesis of schizophrenia, overactivity of the endocannabinoid system may lead to a hyperdopaminergic and hypoglutamatergic state, which may underlie some of the symptoms (Emrich et al., 1997, reviewed in Ujike and Morita, 2004; Laviolette and Grace, 2006). The endocannabinoid hypothesis is supported by multiple lines of evidence. First, the use of large amounts of cannabis and THC may produce psychotic symptoms in normal individuals, including delusions, hallucinations, and cognitive impairment, which resemble schizophrenia (Spencer, 1971; Halikas et al., 1972; Chopra and Smith, 1974; McGuire et al., 1994; Emrich et al., 1997; Johns, 2001; D'Souza et al., 2004). Second, cannabis and THC may worsen psychotic symptoms in schizophrenic patients, contribute to poor outcome, increase the possibility of relapse, and decrease the effectiveness of antipsychotic drugs (Breakey et al., 1974; Treffert, 1978; Negrete, 1989; Turner and Tsuang, 1990; Linszen et al., 1994; Martinez-Arevalo et al., 1994; Voruganti et al., 2001; D'Souza et al., 2005). Third, the use of cannabis may precipitate the onset of schizophrenia in individuals susceptible to psychosis (Andreasson et al., 1987; Miller et al., 2001). Fourth, postmortem radioligand studies document increased CB_1 receptor density in the dorsolateral and anterior cingular regions and subregions of the prefrontal cortex in schizophrenia (Dean et al., 2001; Zavitsanou et al., 2004; Newell et al., 2006). Fifth, the levels of anandamide are increased in cerebrospinal fluid or blood from schizophrenic patients (Leweke et al., 1999; De Marchi et al., 2003; Giuffrida et al., 2004). Sixth, treatment with neuroleptics appears to normalize the imbalance in endocannabinoid signaling in blood cells in schizophrenic patients (De Marchi et al., 2003) and also decreases CB_1 receptor binding in the rat nucleus accumbens (Sundram et al., 2005). Last, the hebephrenic type of schizophrenia shows a strong association with polymorphisms in the *CNR1* gene encoding CB_1 receptors (Leroy et al., 2001; Ujike et al., 2002).

Taken together, the above evidence suggests that the endocannabinoid system may be a novel therapeutic target in schizophrenia. It is also tempting to speculate that CB_1 antagonists may be beneficial against some, most likely the negative, symptoms of the disease. Some preclinical and clinical evidence also suggests that cannabidiol may have antipsychotic potential (reviewed in Zuardi et al., 2006).

b. Anxiety and depression. Mood disorders such as generalized anxiety or panic disorder, major depressive disorder and bipolar disorder (manic depressive illness) are very common, often serious, and potentially life-threatening conditions. More than 20% of the adult population experiences a mood disorder at some point during their life. In up to 15% of individuals with major depressive disorder the cause of death is suicide. According to a World Health Organization forecast, by the year 2020 depression will become the second leading cause of premature death and disability worldwide (Pacher and Kecskeméti, 2004). Although significant advances have been made in the treatment of mood disorders during the past decades, ~30% of the population do not respond to current therapies, and the search for novel pharmacological approaches continues (reviewed in Pacher and Kecskeméti, 2004).

Many of the psychological effects of cannabis and THC are biphasic and bidirectional, depending on mode of administration, dose, personality, time frame, degree of tolerance, and various other environmental and individual factors (Paton and Pertwee, 1973; Ashton et al., 1981, 2005; Viveros et al., 2005). The acute effects in normal subjects can range from euphoria, relaxation, excitation, heightened perception, and increased motor activity to dysphoria, anxiety, sedation, perceptual distortion, and incoordination. THC, under certain conditions and at certain doses, exerts anxiolytic, antidepressant, and hypnotic effects in patients suffering from pain associated with cancer or multiple sclerosis and improves mood and general well-being in normal subjects (Regelson et al., 1976; Glass et al., 1980; Ashton et al., 1981; Fabre and McLendon, 1981; Ilaria et al., 1981; Martyn et al., 1995; Ashton, 1999; Wade et al., 2003). However, under different conditions and at higher doses, cannabis or THC can produce dysphoric reactions, anxiety, panic paranoia, and psychosis (Spencer, 1971; Halikas et al., 1972; Chopra and Smith, 1974; Ashton et al., 1981, 2005; McGuire et al., 1994; Emrich et al., 1997; Johns, 2001; Patton et al., 2002; Tournier et al., 2003;

Dannon et al., 2004; D'Souza et al., 2004; reviewed in Hollister, 1986; Hall and Solowij, 1998).

CBD also possesses anxiolytic, antipsychotic and anticonvulsant properties, which are not mediated by classic cannabinoid receptors (Carlini et al., 1975; Consroe and Wolkin, 1977; Consroe et al., 1981; Zuardi et al., 1982, 1995, 2006; Ames and Cridland, 1986; Martin et al., 1987; Guimaraes et al., 1990, 1994; reviewed in Mechoulam et al., 2002c; Grotenhermen, 2003; Long et al., 2006). The mode of action of CBD is not completely understood; it may involve blockade of anandamide and serotonin reuptake (Bisogno et al., 2001; McPartland and Russo, 2001), inhibition of the enzymatic hydrolysis of anandamide (Mechoulam et al., 2002), or an interaction with as yet unidentified receptors (Járai et al., 1999; Pertwee et al., 2002).

Animal studies yielded further support to the biphasic and bidirectional effects of cannabinoids on anxiety, with low doses being anxiolytic and high doses being anxiogenic. Indeed, low doses of CP55,940 (Genn et al., 2003; Marco et al., 2004), nabilone (Onaivi et al., 1990), and THC (Berrendero and Maldonado, 2002) exerted anxiolytic-like effects in the light-dark crossing test and in the elevated plus-maze in adult rodents. Low-dose CP55,940 was also anxiolytic in other models of anxiety in adult, juvenile, or infant rodents (Romero et al., 2002a; Borcel et al., 2004; Genn et al., 2004). In contrast, at medium to high doses, CP55,940 or HU-210 displayed anxiogenic effects in the same or other experimental paradigms in adult as well as in juvenile or infant animals (McGregor et al., 1996a,b; Rodriguez de Fonseca et al., 1996; Giuliani et al., 2000; Arevalo et al., 2001; Marin et al., 2002; Romero et al., 2002; Genn et al., 2003;2003, 2004;Marin Marco et al., 2004). Although several hypotheses have been proposed to explain the biphasic effects of cannabinoids on anxiety, including distinct receptors (Haller et al., 2004a,b) or neuroanatomically separated CB_1 receptors with a differential sensitivity to the anxiolytic versus anxiogenic effects of cannabinoids, these need to be confirmed in future studies (reviewed in Viveros et al., 2005).

The high level of CB_1 receptors in the hippocampus, amygdala, and prefrontal and anterior cingular cortex, key regions in the regulation of anxiety, may suggest that the endocannabinoid system plays a role in the control of anxiety (Herkenham et al., 1990, 1991a,b; Glass et al., 1997b; Katona et al., 2001; Hájos and Freund, 2002; Tzavara et al., 2003; Pistis et al., 2004). Further support of this theory came from studies using CB_1 receptor antagonists or CB_1 receptor knockout mice. SR141716 produced anxiogenic effects in the elevated plus-maze and the defensive withdrawal tests in adult rats (Navarro et al., 1997; Arevalo et al., 2001). Furthermore, SR141716 not only reversed the anxiolytic effects of the CB_1 agonist CP55,940 but also was anxiogenic in the ultrasonic vocalization test in rat pups when administered alone (McGregor et al., 1996a). In contrast, Haller et al. (2002) found SR141716 to be anxiolytic in the plus-maze in mice, but this effect was not mediated by CB_1 receptors as indicated by its presence in CB_1 knockout mice. Furthermore, another selective CB_1 receptor antagonist, AM251, increased anxiety-like behavior in wild-type mice but had no effect in the knockouts, in support of a CB_1 receptor-mediated anxiolysis. As discussed before, SR141716, but not AM251, also inhibits a CB_1-like receptor that mediates presynaptic inhibition of glutamate release in the hippocampus (Hájos and Freund, 2002). Thus, the findings of Haller et al. (2002) could suggest that the anxiolytic effect of SR141716 is mediated by such a CB_1-like receptor, activation of which would be anxiogenic.

CB_1 knockout mice displayed increased anxiogenic responses in the light-dark box, plus-maze, and social interaction tests, an increased aggressive response in the resident-intruder test, and marked alterations in the hypothalamic-pituitary-adrenal (HPA) axis coupled with impaired action of known anxiolytic drugs such as buspiron and bromazepam (Haller et al., 2002, 2004b; Martin et al., 2002; Urigüen et al., 2004). However, Marsicano et al. (2002) were unable to demonstrate anxiogenic-like response in CB_1 knockout mice in the plus-maze. This may be related to differences in the genetic background of the CB_1 knockout mice used and/or different experimental conditions. The importance of the latter is also indicated by the confounding effect of stress on anxiogenic behaviors and their modulation by endocannabinoids (Haller et al., 2004a; Patel et al., 2005). Stress-induced down-regulation of hippocampal endocannabinoid signaling may contribute to problems in behavioral flexibility and may play a role in the development of perseveratory and ruminatory behaviors in stress-related neuropsychiatric disorders (Hill et al., 2005). Collectively, a majority of evidence supports a role for CB_1 receptors in the control of emotional behavior and suggests the existence of an anxiolytic endocannabinoid tone. Facilitation of such a tone by inhibiting the degradation of endocannabinoids in vivo may be therapeutically exploited, as indicated by the reduced anxiety-like behavior and potent antidepressant-like effects in mice and rats treated with a FAAH or anandamide transport inhibitor and the blockade of this effect by SR141716 or AM281 (Kathuria et al., 2003; Gobbi et al., 2005; Bortolato et al., 2006; Rutkowska et al., 2006).

The mechanisms responsible for the effects of cannabinoids on anxiety-related responses are complex and may involve modulation of numerous neurotransmitter systems. For example, stimulation of CB_1 receptors in rodents activates the HPA axis through the release of CRH (Weidenfeld et al., 1994; Wenger et al., 1997; Martin-Calderon et al., 1998; Manzanares et al., 1999a; Marco et al., 2004), which could account for the anxiogenic effects of high doses of cannabinoids (Rodriguez de Fonseca et al., 1996; Marin et al., 2002). In contrast, there are also examples of negative modulation of HPA

function by endocannabinoids (Di et al., 2003; Patel et al., 2004). Cannabinoids also modulate GABAergic transmission and the release of the peptide cholecystokinin, which may contribute to both anxiolytic and anxiogenic effects (Onaivi et al., 1990; Katona et al., 1999, 2001; Marsicano and Lutz, 1999; Tsou et al., 1999; Beinfeld and Connolly, 2001; Rotzinger and Vaccarino, 2003). Furthermore, cannabinoids enhance the release of endogenous opioids and a functional interplay between the endocannabinoid and opioid systems modulates analgesic responses and is involved in antidepressant-like effects and in various addiction-related processes (Pugh et al., 1997; Manzanares et al., 1999b; Houser et al., 2000; Zimmer et al., 2001; Ghozland et al., 2002). From studies with THC and CP55,940, it appears that μ- and δ-opioid receptors mediate certain anxiolytic effects, whereas activation of κ-opioid receptors leads to increased anxiety (Pugh et al., 1997; Houser et al., 2000; Zimmer et al., 2001; Berrendero and Maldonado, 2002; Ghozland et al., 2002; Marin et al., 2003). There are also interactions between the endocannabinoid and serotonergic systems (Arevalo et al., 2001; Malone and Taylor, 2001; Fride and Shohami, 2002; Marin et al., 2003; Marco et al., 2004; Steffens and Feuerstein, 2004; reviewed in Viveros et al., 2005), although their role in anxiety-like behaviors has not been explored

In contrast to earlier dogma, recent findings indicate that neurogenesis occurs in the adult brain. Furthermore, stress and depression decrease neurogenesis, particularly in the hippocampus, whereas electroconvulsive therapy and chronic treatment with conventional antidepressants increases this process (reviewed in Pacher et al., 2004a). It has been recently demonstrated that the endocannabinoid system drives neural progenitor cell proliferation (Aguado et al., 2005, 2006), and cannabinoids promote neurogenesis (Berghuis et al., 2005; Jiang et al., 2005). Furthermore, CB_1 receptors appear to be required for neuronal survival in the hippocampus (Bilkei-Gorzo et al., 2005). These findings are particularly exciting, as they raise the possibility of a role for endocannabinoids in antidepressive drug action. Indeed, CB_1 receptor density in the hippocampus and hypothalamus is increased by chronic tricyclic antidepressant treatment (Hill et al., 2006), and the amplification of the actions of endocannabinoids by the FAAH inhibitor URB597 was found to produce antidepressant-like effects in the mouse tail-suspension and rat forced-swim tests, without eliciting reward-related effects indicative of addictive potential (Gobbi et al., 2005). It should not be surprising, however, that based on the basis of the bimodal action of cannabinoids on mood and anxiety, a case could be made for the opposite, i.e., for the antidepressive potential of CB_1 *antagonism*. CB_1 antagonists were reported to elicit antidepressant-like behavioral effects in rodents and can increase the synaptic concentration of biogenic amines, much like antidepressants do (reviewed in Witkin et al., 2005). Thus, pharmacological modulation of the endocannabinoid system holds considerable promise in the treatment of both anxiety-related and mood disorders.

The results of a recent study implicated endocannabinoids and CB_1 receptors in the extinction of aversive memories by demonstrating that CB_1 knockout mice show impaired extinction in auditory fear-conditioning tests, and this could be mimicked in wild-type mice by treatment with SR141716 (Marsicano et al., 2002b). These exciting findings raise the possibility that pharmacological amplification of CB_1 signaling, for example, by FAAH inhibitors, may have therapeutic value in obsessive-compulsive disorder or post-traumatic shock syndrome.

9. Insomnia. Insomnia, the most common sleep disorder, is defined as difficulty with the initiation, maintenance, duration, or quality of sleep that results in the impairment of daytime functioning, despite adequate opportunity and circumstances for sleep (Silber, 2005). The cause for insomnia is often not known, but frequently it may be a consequence of a chronic disease associated with pain or depression.

Early studies documented the fact that marijuana and THC affect sleep patterns both in humans (Freemon, 1972, 1982; Pivik et al., 1972; Barratt et al., 1974; Feinberg et al., 1975, 1976) and in experimental animals (Monti, 1977; Buonamici et al., 1982). More recently, Nicholson et al. (2004) have studied the effects of cannabis extracts on nocturnal sleep, early-morning performance, memory, and sleepiness in a placebo-controlled, double-blind, crossover study in eight healthy volunteers. They found that 15 mg of THC was sedative, whereas 15 mg of CBD had alerting properties as it increased wake activity during sleep and counteracted the residual sedative activity of THC (Nicholson et al., 2004).

Anandamide was also found to modulate sleep by increasing slow-wave sleep two and rapid eye movement sleep in a CB_1 receptor-dependent manner in rats (Murillo-Rodriguez et al., 1998, 2001). Moreover, CB_1 receptor expression in the pons of rats was modulated by the light/dark cycle and by sleep (Martinez-Vargas et al., 2003), and endocannabinoids and CB_1 receptors were also implicated in rapid eye movement sleep rebound (Navarro et al., 2003). Interestingly, a recent study has demonstrated that anandamide not only induced sleep but also increased levels of the sleep-inducing substance adenosine in the basal forebrain, and both of these effects were blocked by SR141716 (Murillo-Rodriguez et al., 2003).

Oleamide is a fatty acid amide with a variety of in vitro effects, including inhibition of gap junction-mediated cell-cell communication (Boger et al., 1998a,b), modulation of 5-HT_1, 5-$HT_{2A,C}$, and 5-HT_7 receptors (Thomas et al., 1997, 1999; Hedlund et al., 1999), and modulation of inhibitory ionotropic receptors such as the $GABA_A$ receptor (Coyne et al., 2002). Oleamide accumu-

lates in the cerebrospinal fluid of sleep-deprived cats (Cravatt et al., 1995) and rats (Basile et al., 1999) and induces sleep, an effect which could be blocked by SR141716 (Mendelson and Basile, 1999). Initially, it was suggested that inhibition of anandamide degradation by FAAH rather than the activation of CB_1 receptors was responsible for the sleep-inducing effect of oleamide (Boring et al., 1996; Mechoulam et al., 1997), but this is a matter of dispute (Fowler, 2004; Lees and Dougalis, 2004; Leggett et al., 2004).

Although little is known about the role of the endocannabinoid system in the pathophysiology of sleep disorders, clinical studies uniformly report significantly improved sleep quality in patients taking cannabinoids for symptomatic treatment of multiple sclerosis, cancer, chronic pain, or intractable pruritus. Although psychotropic cannabinoids are unlikely to gain acceptance for the treatment of insomnia, FAAH inhibitors were shown to enhance certain endocannabinoid-mediated behaviors without evidence for addictive properties (Kathuria et al., 2003). The sleep-inducing property of some potent FAAH inhibitors, such as the endogenous lipid 2-octyl γ-bromoacetoacetate (Boger et al., 1998a), could therefore be therapeutically exploited.

10. Nausea and Emesis. Nausea and vomiting can present as symptoms of a variety of diseases or as secondary consequences of chemotherapy or radiotherapy of cancer. It is for this latter indication that THC has gained acceptance as a highly efficacious therapeutic agent, often effective in cases resistant to other, more conventional, medications (reviewed by Martin and Wiley, 2004; Aapro, 2005; Hall et al., 2005). Emesis is thought to involve activation of specific receptors on sensory nerve endings in the gut and also in brainstem regions including the medullary chemoreceptor trigger zone and the lateral reticular formation. Activation of $5\text{-}HT_3$ receptors appears to play a dominant role in acute emesis, whereas activation of NK_1 (substance P) receptors is more important in the delayed emesis after chemotherapy, as indicated by the effectiveness of the respective receptor antagonists in controlling these different stages of the emetic response (Aapro, 2005). Although the mechanism of the antiemetic action of cannabinoids is not quite clear, an interaction with $5\text{-}HT_3$ is suggested by the colocalization of CB_1 and $5\text{-}HT_3$ receptors on GABAergic neurons where they have opposite effects on GABA release (Morales et al., 2004). Also, cannabinoids may directly inhibit $5\text{-}HT_3$-gated ion currents by a mechanism not involving CB_1 receptors (Fan, 1995; Barann et al., 2002). Such a CB_1 receptor-independent effect is also suggested by the ability of cannabidiol, a natural constituent of marijuana which does not bind to the CB_1 receptor, to reduce lithium-induced vomiting in the house musk shrew (Parker et al., 2004). Nevertheless, the involvement of CB_1 receptors is clearly indicated by the ability of SR141716 to reverse the effects of THC and synthetic agonists in suppressing vomiting caused by cisplatin (Darmani, 2001b) or lithium chloride (Parker et al., 2004), or by the ability of these agonist to reverse the emesis elicited by SR141716 in the least shrew (Darmani, 2001a). These latter findings suggest that the emetic circuitry is tonically controlled by endocannabinoids.

In line with such a possibility, a recent human study found an association between chronic marijuana use, which probably results in desensitization of cannabinoid receptors, and cyclical hyperemesis: in the 19 subjects studied, the hyperemetic episodes subsided upon discontinuation of cannabis use and reappeared upon rechallenge with cannabis (Allen et al., 2005). A meta-analysis of 30 randomized comparisons of cannabis (nabilone, dronabinol, or levonantradol) with placebo or standard antiemetics, involving a total of 1366 patients, concluded that cannabinoids are slightly more effective than conventional antiemetics, and the patients prefer them because of their mood enhancing and sedative effects. However, they were also more toxic, with dizziness, dysphoria, hallucinations, and paranoia being the most prominent undesirable side effects (Tramèr et al., 2001). This led to the recommendation to limit the use of cannabinoids as antiemetics to patients with chemotherapy-related sickness, in whom their mood-enhancing effects would be of added benefit.

11. Drug Addiction and Alcohol Disorders. The positive reinforcing effect of natural rewards, such as those derived from eating, drinking, work, or sexual activity, are mediated by the brain's reward circuitry. Neuroanatomically, this circuitry consists of three series of coupled pathways. First-order neurons project from structures in the ventral limbic forebrain (orbitofrontal cortex and anterior cingulate area) to the mesencephalic ventral tegmental area (VTA) where they synapse onto dopaminergic neurons. These second-order neurons project primarily to neurons in the shell of the nucleus accumbens (nAc), but also to cortical areas and to the amygdala. Third-order neurons in the nAc, some of which are GABAergic, project to the ventral pallidum and other regions involved in mediating reward-related behaviors (recently reviewed by Lupica et al., 2004; Gardner, 2005). It is believed that addictive drugs activate or "hijack" the same pathway. Genetic vulnerability to drug addiction has been linked to a functional deficiency in the second-order dopaminergic neurons at their interface with third-order neurons in the nAc (Nestler, 2003). In human subjects prone to addiction, a deficiency in D_2 dopamine receptors in the nAc could be documented by brain imaging (Volkow et al., 1997, 1999).

A common denominator among different addictive drugs interacting with distinct receptors is their ability to activate the mesolimbic dopaminergic reward pathway and increase dopamine levels in the nAc, which is believed to be responsible for their addictive properties (Koob, 1992; Wise, 2004). Similar to other drugs of abuse, THC increases extracellular dopamine levels in

the nAc via activation of CB_1 receptors (Chen et al., 1990; Tanda et al., 1999) and also lowers the reward threshold for electrical brain stimulation (Gardner et al., 1988), a phenomenon known to involve activation of the mesolimbic dopamine system. THC also increases the firing rate of the second-order VTA-nAc dopaminergic neurons via CB_1 but not opiate receptors (French, 1997), and withdrawal from THC increases corticotropin-releasing factor levels in the central nucleus of the amygdala (Rodriguez de Fonseca et al., 1997), another hallmark of drugs of abuse (Koob, 1996).

THC and related synthetic cannabinoid agonists also fulfill the reward-related behavioral criteria for drugs of abuse: they support conditioned place preference (CPP) under appropriate conditions (Lepore et al., 1995; Valjent and Maldonado, 2000; Zangen et al., 2006), they are self-administered intravenously or intracerebrally in a CB_1 antagonist-sensitive manner (Martellotta et al., 1998; Ledent et al., 1999; Braida et al., 2001; Zangen et al., 2006), and they reinstate cocaine-or heroine-seeking behavior in rats previously extinguished from self-administration (De Vries et al., 2001).

An issue of intense interest is the location of the CB_1 receptors mediating these effects. Similar to cannabinoids, opiates also increase the activity of dopaminergic neurons in the VTA. This effect has been shown to result from μ receptor-mediated inhibition of GABA release from the terminals of inhibitory GABAergic interneurons, i.e., through a "disinhibitory" mechanism (Johnson and North, 1992). A similar mechanism has been postulated for cannabinoids by Cheer et al. (2000), who reported that local application of the cannabinoid agonist HU-210 to brain slices containing the VTA increased dopaminergic neuronal activity, which could be blocked by the $GABA_A$ antagonist bicuculline. In line with this, WIN 55,212-2 was found to suppress electrically evoked, but not muscimol-induced, inhibitory postsynaptic currents via CB_1 receptors in brain slices containing the VTA (Szabo et al., 2002). However, cannabinoids also inhibit glutamate release in the VTA, which would have an opposite effect on dopaminergic activity (Melis et al., 2004a). There is evidence for additional sites of action, such as CB_1 receptors on the terminals of GABAergic projection neurons that target $GABA_B$ receptors on VTA dopamine neurons resulting in their disinhibition (Riegel and Lupica, 2004). This pathway may be activated by ethanol, as indicated by the ability of the $GABA_B$ agonist baclofen to antagonize the increase in ethanol drinking caused by WIN 55,212-2 treatment of alcohol-preferring rats (Colombo et al., 2004). Activation of CB_1 receptors on glutamatergic terminals in the nAc was reported to inhibit glutamate release onto GABAergic neurons in the nAc that project to the VTA, which may also result in disinhibition of VTA dopaminergic neurons (Robbe et al., 2001). Indeed, both the VTA and the nAc may be sites of the rewarding effects of cannabinoids, as documented by the propensity of rats to self-administer THC into either site (Zangen et al., 2006).

Regardless of the exact location of presynaptic CB_1 receptors, their natural activation occurs through retrograde transmission, with their endogenous ligands being released from postsynaptic cells (Kreutzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). This mechanism has also been implicated in LTD (Gerdeman et al., 2002; Robbe et al., 2002), a form of synaptic plasticity that can be initiated by drugs of abuse (Thomas et al., 2001), and may be involved in certain features of compulsive drug use (Gerdeman et al., 2003). A further indication that endocannabinoids may be involved in mechanisms of drug reward is findings that the neurochemical and behavioral responses to different classes of drugs of abuse can be inhibited by the CB_1 receptor antagonists. These findings suggests that endocannabinoid activation of CB_1 receptors in the mesolimbic reward pathway may be part of a "common pathway" of drug reward (reviewed in De Vries and Schoffelmeer, 2005; Maldonado et al., 2006). Examples of this are discussed below.

a. Opiates. There is a large body of evidence indicating a reciprocal relationship between the endocannabinoid and endogenous opioid systems in drug dependence (recently reviewed by Fattore et al., 2005; Vigano et al., 2005a,b). This fact is not surprising, given that opioids and cannabinoids have a similar pharmacological profile at both the behavioral level (e.g., analgesia, hypothermia, catalepsy, and motor impairment) and cellular/molecular levels (both CB_1 and opiate μ receptors are predominantly presynaptic, they are coupled to and share the same pool of G_i/G_o proteins, and have an overlapping brain distribution). There are numerous examples for opioid or cannabinoid reward-related effects being inhibited by both CB_1 and opiate μ antagonists (Fattore et al., 2005; Gardner, 2005; Vigano et al., 2005a,b). The mechanisms underlying these reciprocal interactions are not clear, but they may involve heterodimerization of CB_1 and μ opiate receptors, depletion of shared G protein pools and/or utilization of common postreceptor signaling pathways. In addition, the opiate/cannabinoid synergism observed in nAc/striatal neurons appears to require adenosine and A2a receptor signaling (Yao et al., 2006).

Here we will only review evidence that pertains to the potential involvement of endocannabinoids in the addictive, reward-related actions of opioids. Such evidence is based on the ability of pharmacological or genetic ablation of CB_1 receptors to prevent or inhibit opioid effects. CB_1 knockout mice were reported to be unable to acquire morphine self-administration (Ledent et al., 1999; Cossu et al., 2001), to have reduced morphine withdrawal symptoms (Ledent et al., 1999), and not to develop CPP for morphine (Martin et al., 2000). A possible neurochemical correlate of these changes is the lack of morphine-induced dopamine release in the nucleus accum-

bens of CB_1 receptor knockout mice (Mascia et al., 1999), although more recently CB_1 blockade was found to reverse the morphine-induced decrease in ventropallidal GABA overflow without affecting the morphine-induced increase in dopamine release in the nAc (Caillé and Parsons, 2006). Treatment of wild-type mice and rats with a CB_1 antagonist elicits similar phenotypes (Rubino et al., 2000; Mas-Nieto et al., 2001; Navarro et al., 2001, 2004). These observations raise the therapeutic potential of chronic treatment with a CB_1 receptor antagonist in preventing or reversing the development of opiate dependence.

b. Nicotine. Nicotine is the main neuroactive component in tobacco smoke and is responsible for its addictive properties. Nicotine's rewarding effects are mediated by the same mesolimbic dopaminergic pathway that is involved in the rewarding effects of many other addictive drugs (Pontieri et al., 1996). Therefore, it should not be unexpected that there is a positive synergism between nicotine and THC in paradigms used to reveal reinforcing effects (Valjent and Maldonado, 2000). A role of endocannabinoids in the rewarding effects of nicotine is indicated by the absence of nicotine-induced CPP in CB_1 knockout mice (Castane et al., 2002), although the acquisition of nicotine self-administration was not affected by the absence of CB_1 receptors in another study using an acute reinforcement paradigm (Cossu et al., 2001). On the other hand, SR141716 was reported to decrease nicotine operant self-administration (Cohen et al., 2002) and nicotine-induced CPP in rats (Le Foll and Goldberg, 2004; Forget et al., 2006) and also to inhibit nicotine-induced dopamine release in the nucleus accumbens shell (Cohen et al., 2002). SR141716 also inhibited nicotine self-administration sustained by nicotine-associated cues in the absence of nicotine itself (Cohen et al., 2005), and chronic exposure to nicotine was reported to induce endocannabinoid release (Gonzalez et al., 2002). Furthermore, SR141716 abolished the anxiolytic effects of low-dose nicotine in mice and potentiated its anxiogenic effects at higher doses (Balerio et al., 2006). Together, these findings justified testing rimonabant in clinical trials to promote smoking abstinence. Indeed, the results of a recent multicenter phase III clinical trial in the United States indicate that a 10-week treatment of smokers with a daily oral dose of 20 mg of rimonabant with a follow-up period of 42 weeks doubled the odds of quitting smoking, was well tolerated, and also reduced the post-cessation weight gain by >80% (Dale and Anthenelli, 2004).

c. Cocaine. Unlike THC, opiates and nicotine, cocaine does not increase the activity of dopaminergic neurons in the VTA but elevates synaptic levels of dopamine in the nAc by blocking dopamine reuptake at the dopamine transporter (Giros et al., 1996). Therefore it is not surprising that cocaine-induced increases in dopamine in the nAc were found to be unaffected by genetic ablation of CB_1 receptors (Soria et al., 2005). Accordingly, CB_1 receptors do not appear to participate in the acute rewarding properties of cocaine, as indicated by the preserved acute cocaine self-administration and cocaine-induced CPP in CB_1 knockout mice (Martin et al., 2000; Cossu et al., 2001; Lesscher et al., 2005; Soria et al., 2005) or in mice treated with SR141716 (Tanda et al., 2000; De Vries et al., 2001; Caillé and Parsons, 2006). SR141716 treatment also did not affect the threshold-lowering effect of cocaine in the intracranial self-stimulation paradigm, although treatment with WIN 55,212-2 was able to achieve this, suggesting that CB_1 receptor stimulation might inhibit the reinforcing properties of cocaine (Fattore et al., 1999; Vlachou et al., 2003).

Other studies indicate, however, that endocannabinoid activation of CB_1 receptors may mediate the reinforcing effects of cocaine. SR141716 treatment decreased the sensitivity of rats to the reinforcing effects of cocaine in an intracranial self-stimulation paradigm (Deroche-Gamonet et al., 2001). The ability to acquire operant self-administration of cocaine was reduced in CB_1 knockout mice or in SR141716-treated wild-type mice, which also displayed a reduced maximal effort to obtain cocaine infusion in a progressive ratio schedule, compared with untreated wild-type mice (Martin et al., 2000; Soria et al., 2005). Furthermore, prior use of cannabis was found to enhance the "high" elicited by subsequent use of cocaine in humans (Foltin et al., 1993; Lukas et al., 1994) and also to hasten relapse in abstinent former cocaine users (Rawson et al., 1986). Furthermore, a recent genetic study found an association between an (AAT)n triplet repeat polymorphism in the *CNR1* gene encoding the CB_1 receptor with cocaine addiction in an African-Caribbean population (Ballon et al., 2006). Treatment with HU-210 promoted reinstatement of cocaine-seeking behavior in rats, whereas treatment with SR141716 prevented reinstatement (De Vries et al., 2001). Thus, the endocannabinoid system may be involved in the acquisition and consolidation of cocaine addiction as well as in relapse, through mechanisms other than an effect on the cocaine-induced increase in dopaminergic transmission in the nAc. These latter studies also predict the possible effectiveness of rimonabant in the treatment of cocaine addiction.

d. Alcohol. Several lines of evidence indicate the involvement of the endocannabinoid system in alcohol drinking behavior (recently reviewed by Colombo et al., 2005). Chronic alcohol intake increases endocannabinoid levels in the limbic forebrain (Gonzalez et al., 2002) and decreases CB_1 receptor binding and signaling (Basavarajappa and Hungund, 2002). Studies in the late 1990s indicated the effectiveness of SR141716 in reducing voluntary ethanol intake in rodent models of ethanol drinking (Arnone et al., 1997; Colombo et al., 1998b; Freedland et al., 2001), whereas cannabinoid agonists promoted drinking (Gallate et al., 1999; Colombo et al., 2002). Operant self-administration of ethanol and relapse to drinking are also inhibited by SR141716 (Cipp-

itelli et al., 2005; Economidou et al., 2006) and potentiated by chronic exposure to a cannabinoid agonist (Lopez-Moreno et al., 2005).

The possible role of the endocannabinoid system in ethanol preference was further indicated by observations of reduced voluntary ethanol drinking in CB_1 knockout compared with wild-type mice (Hungund et al., 2003; Poncelet et al., 2003; Wang et al., 2003; Lallemand and de Witte, 2004; Naassila et al., 2004; Thanos et al., 2005), although no difference was noted in one study (Racz et al., 2003). Sensitivity to alcohol is inversely related to the chance of becoming an alcoholic among humans (Schuckit, 1997), and the same inverse relationship was noted in CB_1 knockout mice and their wild-type littermates (Naassila et al., 2004). The reduced voluntary ethanol intake in CB_1 knockout mice was associated with reduced alcohol-induced CPP (Houchi et al., 2004; Thanos et al., 2005), a further indication of the role of CB_1 receptors in the rewarding effects of alcohol.

Similar to cannabinoids and other drugs of abuse, alcohol intake can also result in increased dopamine release in the nAc (Weiss et al., 1993; Campbell and McBride, 1995). The reported absence of such release in CB_1 knockout mice and the ability of SR141716 to block ethanol-induced dopamine release in wild-type mice further suggest the involvement of endocannabinoids in the reinforcing effects of ethanol. However, the brain site where ethanol-induced endocannabinoid release and CB_1 receptor activation occur is not yet known. The recent observation that microinjection of SR141716 into the prefrontal cortex of alcohol-preferring AA rats inhibited ethanol self-administration suggests that this region may be one of the sites involved (Hansson et al., 2006). In the same study, FAAH activity and CB_1 signaling were both reduced in the same brain region of AA rats compared with their nonpreferring ANA counterparts, and microinjection of the FAAH inhibitor URB597 increased ethanol self-administration (Hansson et al., 2006). Analogous findings in female FAAH knockout mice are their increased voluntary ethanol intake and decreased alcohol sensitivity (Basavarajappa et al., 2006). These findings suggest that increased anandamide tone secondary to decreased FAAH activity in the prefrontal cortex may be causally linked to high alcohol preference. Such a scenario would be compatible with evidence for an association between problem drug and alcohol use and a missense mutation in the human FAAH gene (Sipe et al., 2002).

A number of mediators have been implicated in the control of appetite for both food and alcohol. In the case of endocannabinoids, the regulation is "unidirectional", i.e., endocannabinoids promote both food intake (see section III.A.3.) and alcohol drinking. Because both food intake and alcohol drinking activate the brain reward pathways, one might postulate that the role of endocannabinoids in promoting drinking behavior would be most prominent in the type of alcoholics who drink for the rewarding effects of alcohol, such as young binge-drinkers. The high alcohol preference of C57BL6 mice and the role of the endocannabinoid system mediating it were found to be age-dependent (Wang et al., 2003), which is compatible with such a possibility. In contrast, the effects of NPY and CRH on food intake and ethanol consumption are bidirectional: NPY increases food intake (Clark et al., 1984) but reduces ethanol consumption (Thiele et al., 1998), whereas CRH is anoretic (Britton et al., 1982) but promotes ethanol drinking (George et al., 1990). The effects of NPY and CRH on alcohol preference correlate with their effects on anxiety-like behaviors, NPY being anxiolytic (Heilig et al., 1989) and CRH being anxiogenic (Koob and Thatcher-Britton, 1985). We would predict that CB_1 antagonists will be more effective in reducing the drive to drink in younger people who drink for the rewarding effects of alcohol, whereas CRH antagonists or NPY agonists would be more effective in older, chronic alcoholics who more likely drink to suppress the negative affect and anxiety of alcohol withdrawal. This hypothesis may be tested by appropriately designed clinical trials. Studies to test the safety and efficacy of rimonabant in the treatment of alcoholism and alcohol abuse are currently underway at the National Institute on Alcohol Abuse and Alcoholism.

e. Psychostimulants. 3,4-Methylenedioxymethamphetamine (MDMA, Ecstasy) is a psychostimulant abused for its euphorigenic and stimulant properties, and it is often used in combination with marijuana. Intracerebral self-administration of MDMA was found to be reduced in the presence of the cannabinoid agonist CP55,940 and increased after treatment with SR141716. These findings were interpreted to indicate synergism between the reinforcing effects of cannabinoids and MDMA and a reduction in the motivational value of MDMA by CB_1 blockade (Braida and Sala, 2002). In another study, the authors found that SR141716 blocked MDMA-induced CPP (Braida et al., 2005). Amphetamine-induced long-term synaptic depression in the amygdala could be blocked by the CB_1 antagonist AM251, mimicked by the agonist WIN 55,212-2, and occluded by the transport inhibitor AM404, suggesting that amphetamine-induced LTD and related behavioral effects may be mediated via endocannabinoid release (Huang et al., 2003). Together, these findings suggest that CB_1 antagonists may be of value in the treatment of addiction to psychostimulants, including amphetamine and MDMA.

D. Cardiovascular and Respiratory Disorders

Besides their well known neurobehavioral and immunological actions, cannabinoids and their endogenous and synthetic analogs exert important cardiovascular effects. The underlying mechanisms are complex, involving direct effects on the vasculature (Gebremedhin et al., 1999; Járai et al., 1999; Wagner et al., 2001b; Wagner et al., 2005) and myocardium (Bonz et al., 2003; Maslov et

al., 2004; Sterin-Borda et al., 2005), as well as modulation of autonomic outflow through sites of action in the central (Niederhoffer and Szabo, 2000; Pfitzer et al., 2004) and the peripheral nervous systems (Ishac et al., 1996; Malinowska et al., 1997; Szabo et al., 2001; Niederhoffer et al., 2003). As for endogenous cannabinoids, their effects are also complicated by their rapid metabolism, which liberates arachidonic acid that can be further metabolized into vasoactive prostanoids (reviewed in Mechoulam et al., 1998; Kunos et al., 2000; Randall et al., 2002; Ralevic et al., 2002).

Studies to date indicate that CB_1 receptors are much more important than CB_2 receptors in cardiovascular regulation, the latter so far being implicated only in ischemic preconditioning and ischemia/reperfusion (I/R) injury of the myocardium (see below). CB_1 receptors have been detected in the human, rat, and mouse myocardium where they mediate negative inotropy (Bonz et al., 2003; Bátkai et al., 2004b; Pacher et al., 2004b, 2005a,b,d; Engeli et al., 2005; Wagner et al., 2005) and also in vascular tissues (Gebremedhin et al., 1999; Liu et al., 2000), where their activation leads to vasodilation, and both of these effects appear to be involved in the hypotensive effect of anandamide (Wagner et al., 2001a,b; Bátkai et al., 2004a,b; Pacher et al., 2004b, 2005a,b,d) in anesthetized rodents. Sympathetic nerve terminals contain presynaptic CB_1 receptors, stimulation of which inhibits norepinephrine release (Ishac et al., 1996), which contributes to the bradycardic effects of anandamide in vivo (Wagner et al., 2001b). Anandamide-induced cardiovascular depressor effects are devoid of a centrally mediated component (Varga et al., 1996), in contrast to the effects of certain synthetic cannabinoids, which cause centrally mediated sympathoexcitation (Niederhoffer and Szabo, 2000; Gardiner et al., 2001, 2002b).

The vasorelaxant effect of endocannabinoids and synthetic cannabinoids in vitro are complex and display tissue and interspecies differences. They may involve CB_1 and $TRPV_1$ receptor- and NO-mediated or NO-independent mechanisms and also as yet undefined endothelial site(s) of action. A detailed discussion of these in vitro vasodilatory effects can be found in recent reviews (Hillard, 2000; Kunos et al., 2000, 2002; Ralevic et al., 2002; Randall et al., 2002, 2004; Begg et al., 2005; Pacher et al., 2005a,b) and is beyond the scope of this review.

Compared with the growing body of information on the vascular effects of cannabinoids, less is known about cannabinoid-induced direct cardiac effects. Anandamide, *R*-methanandamide, and HU-210 dose dependently decrease contractile performance in isolated, electrically paced human atrial muscle, an effect blocked by the potent CB_1 antagonist AM251, whereas the involvement of CB_2 receptors, NO, or prostanoids could be excluded (Bonz et al., 2003). HU-210 also decreased left ventricular developed pressure in isolated perfused rat hearts through CB_1 receptor activation (Maslov et al., 2004; Krylatov et al., 2005). Another study using isolated, perfused, rat Langendorff heart preparations to study the effects of anandamide, *R*-methanandamide, and palmitoylethanolamide on coronary perfusion pressure and left ventricular developed pressure suggested the involvement of a cardiac site of action distinct from CB_1 and CB_2 receptors (Ford et al., 2002).

Several studies have examined the in vivo hemodynamic effects of endocannabinoids and their synthetic analogs in rodents (recently reviewed in Begg et al., 2005; Pacher et al., 2005a,b). Intravenous administration of anandamide causes a triphasic blood pressure response in anesthetized mice and rats, in which a prolonged hypotensive effect (phase III) is preceded by a transient, vagally mediated, fall in heart rate, cardiac and contractility, and blood pressure and an increase in total peripheral resistance (phase I) followed by a brief, pressor response (phase II) associated with increased cardiac contractility (Varga et al., 1995; Lake et al., 1997b; Pacher et al., 2004b, 2005d). Inhibition of the phase I bradycardic response by $TRPV_1$ receptor antagonists in rats (Malinowska et al., 2001) and the absence of both phase I and phase II responses in $TRPV_1^{-/-}$ mice (Pacher et al., 2004) imply that these components are mediated by $TRPV_1$ receptors. Additional central and vascular mechanisms may also be involved in the brief pressor response (phase II) in anesthetized rats (Kwolek et al., 2005). The third, prolonged hypotensive phase (phase III) is characterized by marked decreased cardiac contractility and slightly decreased total peripheral resistance, and it lasts up to 10 min in anesthetized mice (Pacher et al., 2004b, 2005d), similar to the hypotensive effect previously described in anesthetized but not conscious rats (Stein et al., 1996; Varga et al., 1996; Lake et al., 1997a,b; Gardiner et al., 2002a; Bátkai et al., 2004b) and also observed with synthetic cannabinoids (Vidrio et al., 1996; Lake et al., 1997a; Pacher et al., 2005d).

The anandamide-induced phase III hypotension and decreased cardiac contractility, as well as similar hemodynamic responses to synthetic cannabinoids, are mediated by CB_1 receptors. First, these effects are prevented or reversed by selective CB_1 antagonists both in normal rodents (Varga et al., 1995, 1996; Calignano et al., 1997; Pacher et al., 2004b, 2005a,d) and in mice lacking FAAH, which exhibit increased sensitivity to hypotensive and cardiodepressant effects of anandamide (Pacher et al., 2005d). Second, there is a positive correlation between the concentrations of various cannabinoid agonists in producing half-maximal hypotensive and bradycardic responses (EC_{50}) and in their affinity constants for binding to CB_1 receptors in the brain (Lake et al., 1997a). Third, cannabinoid-induced hypotension and bradycardia are absent in mice lacking the CB_1 receptor (Járai et al., 1999; Ledent et al., 1999). The involvement of the endocannabinoid system in various cardiovascular disorders is reviewed below.

1. Hypertension. Chronic use of cannabis in humans as well as both acute and prolonged administration of THC to experimental animals elicits a long-lasting decrease in blood pressure and heart rate (Rosenkratz and Braude, 1974; Benowitz and Jones, 1975), whereas the acute effect of smoking cannabis usually increases heart rate with no consistent change in blood pressure (Kanakis et al., 1976). In a recent study conducted in 63 male cannabis smokers, 22% of subjects experienced symptomatic hypotension, which could be reversed by the administration of 30 or 90 mg but not lower doses of rimonabant, indicating that CB_1 receptors mediate the hypotensive effect of cannabis smoking in humans (Gorelick et al., 2006).

More than three decades ago, several studies explored the potential use of cannabinoids to treat hypertension (Birmingham, 1973; Archer, 1974; Varma and Goldbaum, 1975; Adams et al., 1977; Crawford and Merritt, 1979; Zaugg and Kyncl, 1983). Unfortunately, the initial high anticipation was tempered by a report of the development of rapid tolerance to the hypotensive and bradycardic effects of THC (Adams et al., 1976) and by the failure to separate the cardiovascular and neurobehavioral effects of cannabinoids. Albeit a later study in spontaneously hypertensive rats (SHR) demonstrated no tolerance to the same effects during a 10-day treatment period (Kosersky, 1978), interest in this issue had vanished for the next two decades.

As with many other effects of marijuana, the discovery of endocannabinoids has focused attention on their possible role in cardiovascular regulation. Studies with SR141716 indicated that the hypotensive/bradycardic effects of exogenous anandamide, THC, and potent synthetic cannabinoids are mediated by CB_1 receptors (Varga et al., 1995; Lake et al., 1997a). CB_1 receptor knockout mice have normal blood pressure (Járai et al., 1999; Ledent et al., 1999) and the blood pressure of normotensive mice and rats is unaffected or slightly reduced by CB_1 antagonists (Varga et al., 1995; Lake et al., 1997a;Varga Bátkai et al., 2004b). In anesthetized rats, anandamide elicits only a modest and short-lasting hypotensive response (Varga et al., 1995; Lake et al., 1997a), whereas in conscious normotensive rats it has no hypotensive effect at all (Stein et al., 1996; Lake et al., 1997b; Gardiner et al., 2002). Furthermore, inhibitors of anandamide transport or FAAH do not lower blood pressure in normotensive animals (Calignano et al., 1997; Bátkai et al., 2004b), and mice deficient in FAAH have normal baseline hemodynamic characteristics and baroreceptor reflex function (Pacher et al., 2005d). As pointed out by a recent editorial (Awumey et al., 2005), these observations indicate a lack of involvement of endogenous cannabinoids in cardiovascular regulation under normal conditions.

In contrast, a number of observations indicate that endocannabinoids *are* involved in cardiovascular regulation in hypertension. Both THC (Kosersky, 1978) and anandamide (Lake et al., 1997b, Bátkai et al., 2004b) induce larger and longer lasting hypotension in anesthetized SHR compared with normotensive controls, and the hypotensive effect of anandamide is preserved in conscious SHR (Lake et al., 1997b). Interestingly, inhalation of THC also resulted in a greater and longer lasting decrease of arterial blood pressure in hypertensive compared with normotensive individuals (Crawford and Merritt, 1979). By using a sophisticated pressure-volume analysis system, the hemodynamic effects of cannabinoid agonists and antagonists were evaluated in three different models of experimental hypertension (Bátkai et al., 2004b). In anesthetized SHR, the CB_1 antagonists AM251 and SR141716 both caused marked and sustained further increases in blood pressure and cardiac contractility (Fig. 5). Conversely, preventing the degradation or uptake of endogenous anandamide by treatment with the FAAH inhibitor URB597 or the transport inhibitor OMDM2 reduced blood pressure, cardiac contractility, and vascular resistance to levels observed in normotensive controls, and these effects were prevented by pretreatment with a CB_1 antagonist. Similar effects were seen in Dahl salt-sensitive rats and rats with angiotensin II-induced hypertension, whereas in the respective normotensive controls the same parameters remained unaffected by any of these treatments (Bátkai et al., 2004b) (Fig. 5). Anandamide and HU-210 induced more pronounced and longer lasting hypotension in SHR than in WKY rats. Unexpectedly, decreased cardiac contractility rather than a reduction in peripheral resistance was primarily responsible for the antihypertensive effect of anandamide, which was fully prevented by CB_1 antagonists, but was unaffected by the $TRPV_1$ antagonist capsazepine. In the same study, the expression of CB_1 receptors was found to be increased in the myocardium and the aortic endothelium of SHR compared with WKY rats.

These findings point to the existence of an endocannabinoid tone in hypertension that limits the elevation of blood pressure and cardiac contractile performance through tonic activation of cardiac and probably vascular CB_1. A possible underlying mechanism is the observed up-regulation of cardiac and vascular CB_1 in SHR compared with their normotensive controls, although increased coupling of these CB_1 receptors may also contribute to the augmented sensitivity to the cardiovascular effects of anandamide (Bátkai et al., 2004b). A proposed alternative mechanism would involve up-regulation of vascular $TRPV_1$ receptors in hypertension, based on the reported ability of capsazepine to partially inhibit the hypotensive effect of anandamide and *R*-methanandamide in hypertensive but not in normotensive rats (Li et al., 2003; Wang et al., 2005). However, capsazepine is known to have nonspecific effects even at low concentrations (Ray et al., 2003), and up-regulation of $TRPV_1$ cannot account either for the increased hypotensive potency of HU-210 (Bátkai et al., 2004b), which

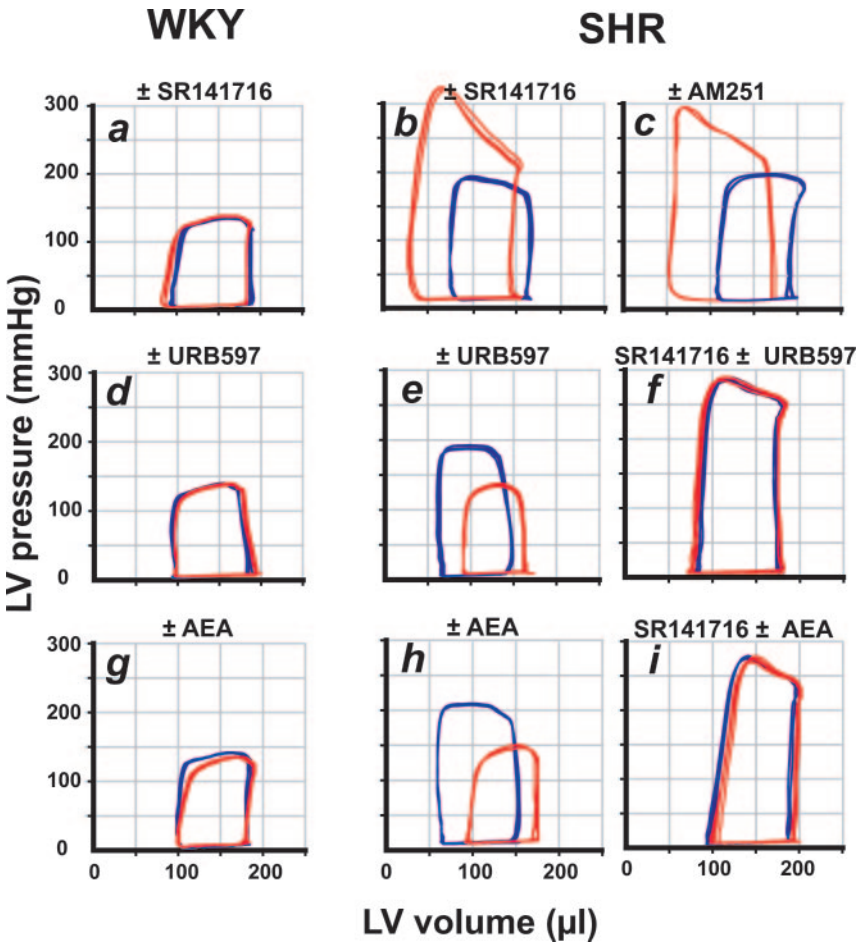


FIG. 5. Effects of anandamide, URB597, SR141716, and AM251 on left ventricular (LV) function in normotensive and spontaneously hypertensive rats. Representative left ventricular pressure-volume (PV) loops from WKY rats (a, d, and g) and SHR (b, c, e, f, h, and i) before (black) and after (red) treatment with indicated agents or their combinations. A leftward shift of PV loops and an increase in amplitude (pressure) indicate increased LV contractility, whereas a rightward shift and decrease in amplitude indicate decreased LV function. Experiments were repeated in three more animals in each treatment group with similar results. AEA, anandamide. Reproduced with permission from Bátkai et al. (2004) *Circulation* **110:**1996–2002; © Lippincott Williams and Wilkins.

is not a ligand for $TRPV_1$ receptors (Zygmunt et al., 1999), or for the dominant cardiac component in the hypotensive effect of exogenous or endogenous anandamide (Bátkai et al., 2004b). Also, physiological concentrations of endogenous anandamide are at least an order of magnitude lower than the micromolar concentrations required to activate $TRPV_1$ receptors.

A practical implication of these findings is that enhancing endocannabinoid tone by blocking the enzymatic degradation or cellular uptake of anandamide could be a novel therapeutic approach in the treatment of hypertension. Such a strategy has a number of desirable features: 1) unlike the generalized activation of CB_1 receptors by direct acting agonists, inhibition of FAAH causes a more restricted profile of cannabinoid-like effects with no indication of psychoactivity (Kathuria et al., 2003; Gobbi et al., 2005), probably related to the discrete distribution of FAAH in the brain; 2) FAAH or transport inhibitors have no hemodynamic effects under normotensive conditions, which predicts the absence of postural hypotension or other side effects; and 3) having a major effect on the inappropriately increased cardiac contractility, such treatment may be effective in reversing the cardiac hypertrophy that usually accompanies chronic hypertension.

2. Circulatory Shock. The profound hypotension that can be elicited through pharmacological activation of CB_1 receptors (Lake et al., 1997a) triggered numerous studies to investigate the role of the endocannabinoid system in the hypotension associated with various forms of shock, including hemorrhagic (Wagner et al., 1997; Cainazzo et al., 2002), endotoxic (Varga et al., 1998; Wang et al., 2001; Liu et al., 2003a; Bátkai et al., 2004a; Gardiner et al., 2005; Kadoi et al., 2005), and cardiogenic shock (Wagner et al., 2001a, 2003), and the shock associated with necrotizing pancreatitis (Matsuda et al., 2005). Initial studies demonstrated that the putative CB_1 receptor antagonist SR141716 prevented or reversed the hypotension associated with hemorrhagic, endotoxic, and cardiogenic shock (Wagner et al., 1997, 2001a,b; Varga et al., 1998). Likewise, SR141716 reversed the hypotension associated with advanced liver

cirrhosis (Bátkai et al., 2001; Ros et al., 2002), which is possibly secondary to the endotoxemia frequently found in patients with late-stage cirrhosis (Lumsden et al., 1988). Observations that circulating macrophages and platelets from endotoxemic or cirrhotic animals or humans had elevated levels of endocannabinoids and, when isolated and injected into normal rats, these cells elicited SR141716-sensitive hypotension also pointed toward the involvement of CB_1 receptors in many of these conditions (Wagner et al., 1997; Varga et al., 1998; Bátkai et al., 2001; Maccarrone et al., 2001, 2002; Ros et al., 2002; Liu et al., 2003a).

Several recent reports demonstrated that anandamide and some atypical cannabinoids can cause both cardiodepressant and vasodilatory effects via as-yet-undefined receptors sensitive to inhibition by SR141716 but not by AM251 (Járai et al., 1999; Ford et al., 2002; Ho and Hiley, 2003; O'Sullivan et al., 2004b), a selective CB_1 antagonist equipotent with SR141716 (Gatley et al., 1997). A recent study compared the effects of SR141716 and AM251 in rats on the acute hypotensive effect of bacterial endotoxin (LPS) administered as an intravenous bolus. Hypotension in this model is fully attributable to the decreased cardiac contractility, whereas peripheral vascular resistance is increased, indicating vasoconstriction (Biber et al., 1988; Cheng et al., 2003). Using this model, the cardiodepressant and hypotensive effects of LPS were inhibited by SR141716 but not by AM251. Furthermore, LPS induced SR141716-sensitive hypotension in wild-type mice and in mice deficient in CB_1 or both CB_1 and CB_2 receptors, suggesting that receptors distinct from CB_1 or CB_2 are primarily responsible for the observed hypotension (Bátkai et al., 2004a). Interestingly, another recent study has demonstrated that the CB_1-selective cannabinoid antagonist AM281 prevented the hemodynamic changes induced by acute LPS injection in rats (Kadoi et al., 2005a). Other results indicate that endocannabinoids may also contribute to endotoxin-induced hypotension indirectly, through CB_1-mediated prejunctional inhibition of sympathoexcitation (Godlewski et al., 2004). In a different shock model in which continuous infusion of LPS in conscious rats causes marked peripheral vasodilatation and increased cardiac output, AM251 attenuated the tachycardic and hind quarter vasodilator effects of LPS. This result was attributed to modulation of β-adrenergic vasodilation, rather than suppression of a direct vasodilator effect by endocannabinoids (Gardiner et al., 2005). Interestingly, in a recent study, Matsuda et al. (2005) demonstrated that AM251 improved mean arterial pressure and survival rate in models of severe acute necrotizing pancreatitis without affecting inflammatory changes, which suggests the involvement of cardiac or vascular CB_1 receptors in the hypotension associated with this condition.

In hemorrhagic, cardiogenic, and endotoxic shock, the cannabinoid agonists HU-210, WIN 55,212-2, and THC improved endothelial function and/or survival (Wagner et al., 1997, 2001a, 2003; Varga et al., 1998; Smith et al., 2000, 2001). Surprisingly, the use of cannabinoid receptor antagonists, including SR141716, AM281, AM251, and SR144528, also leads to survival benefits in endotoxic and septic shock or necrotizing pancreatitis (Varga et al., 1998; Smith et al., 2000, 2001; Cainazzo et al., 2002; Kadoi et al., 2005a,b; Matsuda et al., 2005). In contrast, CB_1 receptor blockade increased mortality in hemorrhagic (Wagner et al., 1997) and cardiogenic shock (Wagner et al., 2001a, 2003), despite the increase in blood pressure. In these latter conditions, endocannabinoid-mediated vasodilation may have survival value through improving tissue oxygenation by counteracting the excessive sympathetic vasoconstriction triggered by hemorrhage or myocardial infarction, and this would be removed by CB_1 blockade. In contrast, CB_1 blockade may improve survival in endotoxic shock by preventing the primary hypotensive response to LPS (reviewed in Kunos et al., 2000; Hiley and Ford, 2003, 2004; Pacher et al., 2005a,c).

It should also be kept in mind that in most of the above conditions, hemodynamic changes are triggered by overwhelming inflammatory reaction, increased oxidative stress, and activation of downstream effector pathways, eventually leading to cardiovascular dysfunction and failure (reviewed in Evgenov and Liaudet, 2005; Pacher et al., 2005e). Therefore, the well known immune-modulatory, anti-inflammatory, and antioxidant effects of cannabinoids should not be overlooked in these conditions. Indeed, endocannabinoids and synthetic cannabinoid agonists decrease inflammatory cytokine release in endotoxin-stimulated cells and in endotoxin-challenged animals (reviewed in Walter and Stella, 2004; Klein et al., 2005). Surprisingly, SR141716 and the CB_2 antagonist SR144528 were also reported to have anti-inflammatory effects (Smith et al., 2000, 2001), which may be attributed to their inverse agonist properties or to $CB_{1/2}$ receptor-independent mechanisms (reviewed in Begg et al., 2005; Pertwee, 2005b,c).

Collectively, it appears that both cannabinoids and antagonists of cannabinoid receptors may exert some beneficial effects in various rodent shock models. Further studies should establish the specificity of these effects and the relevance to various forms of circulatory shock in humans.

3. Myocardial Reperfusion Injury. The endocannabinoid system has been implicated in endotoxin-induced preconditioning against myocardial I/R injury (Lagneux and Lamontagne, 2001). In this study, the effects of 90 min of low-flow ischemia followed by 60 min of reperfusion at normal flow were compared in isolated hearts from rats pretreated with LPS or saline. Endotoxin pretreatment enhanced functional recovery on reperfusion and reduced infarct size compared with controls, and pretreatment with the CB_2 antagonist SR144528 but not the CB_1 antagonist SR141716 abolished the benefi-

cial effects of preconditioning (Lagneux and Lamontagne, 2001). In a follow-up study, SR144528 but not SR141716 also abolished the infarct size-reducing effect of preconditioning induced by heat stress (Joyeux et al., 2002). These initial studies have suggested that the protection was mediated by endocannabinoids acting on CB_2 receptors. In preconditioning induced by a brief period of ischemia (5 min), either CB_2 or CB_1 receptor blockade abolished the protection, and both CB_1 and CB_2 receptors were implicated in the preservation of endothelium-dependent, 5-HT-induced vasodilation by ischemic preconditioning (Bouchard et al., 2003). Perfusion of isolated rat hearts with PEA or 2-AG but not anandamide afforded protection against ischemia by improving myocardial recovery and decreasing myocardial damage and infarct size (Lepicier et al., 2003). The cardioprotective effect of both PEA and 2-AG were completely blocked by SR144528, whereas SR141716 partially inhibited the effect of 2-AG only (Lepicier et al., 2003). Likewise, the selective CB_1 agonist ACEA and the selective CB_2 agonist JWH-015 both reduced infarct size in this model, and the CB_2 receptor-mediated cardioprotection by PEA involved activation of p38/extracellular signal-regulated kinases 1 and 2 and protein kinase C (Lepicier et al., 2003). In another study using isolated perfused rat hearts subjected to ischemia and reperfusion, reduction of the infarct size by anandamide could be equally well antagonized by CB_1 or CB_2 antagonists but could not be mimicked by selective CB_1 or CB_2 agonists, suggesting the involvement of a site distinct from CB_1 or CB_2 receptors (Underdown et al., 2005).

Others have used whole animal models of I/R injury induced by coronary occlusion/reocclusion in anesthetized rats. In this model, anandamide and HU-210 both decreased the incidence of ventricular arrhythmias and reduced infarct size through activation of CB_2 but not CB_1 receptors (Krylatov et al., 2001, 2002a,b,c; Ugdyzhekova et al., 2001, 2002). The moderately CB_2-selective agonist WIN 55,212-2 also reduced the extent of leukocyte-dependent myocardial damage in a more recent mouse study of myocardial I/R in vivo. This effect was abolished by the selective CB_2 receptor antagonist AM630 but was unaffected by AM251 (Di Filippo et al., 2004). In summary, evidence to date indicates that endocannabinoids protect against myocardial ischemic injury models predominantly via CB_2 receptors.

4. Atherosclerosis. Chronic inflammation and the associated oxidative-nitrosative stress are key players in atherosclerosis and cardiovascular aging, and pharmacological modulation of these processes could be of therapeutic benefit (reviewed in Csiszar et al., 2005; Libby and Theroux, 2005). Using the apolipoprotein E knockout mouse model of atherosclerosis, Steffens et al. (2005) reported that orally administered THC significantly inhibited disease progression. Furthermore, CB_2 receptor expressing immune cells were present both in human and mouse atherosclerotic plaques, lymphoid cells isolated from THC-treated mice had diminished proliferation capacity and decreased interferon-γ production, and THC inhibited macrophage chemotaxis in vitro. Most importantly, all of these effects were completely blocked by a selective CB_2 receptor antagonist, suggesting that targeting CB_2 receptors may offer a new approach in the treatment of atherosclerosis (Roth, 2005; Steffens et al., 2005).

5. Asthma. The effect of marijuana on airway functions was among the first to be explored for potential therapeutic benefit (reviewed in Lemberger, 1980; Tashkin et al., 2002). Smoking marijuana and ingesting THC were both found to increase airway conductance in normal, healthy subjects (Tashkin et al., 1973; Vachon et al., 1973), and these effects lasted longer than the bronchodilator effect of the β-adrenergic agonist isoproterenol. Bronchodilation induced by smoked marijuana and oral THC was also documented in subjects with mild to moderate asthma and in asthmatic patients with methacholine- or exercise-induced bronchoconstriction (Tashkin et al., 1974, 1975). Bronchodilation without side effects was observed in asthmatic patients after a low dose (0.2 mg) of nebulized THC (Williams et al., 1976; Hartley et al., 1978). In contrast, aerosols containing larger doses of THC (5–20 mg) caused paradoxical bronchoconstriction attributed to local irritation (Tashkin et al., 1977). In another study of normal and asthmatic subjects, orally administered THC elicited only minimal and inconsistent bronchodilation associated with significant CNS side effects (Abboud and Sanders, 1976). Nevertheless, most of these initial observations had suggested some therapeutic benefit of using cannabinoids in asthma.

As for the mechanisms underlying THC-induced bronchodilation, the potential involvement of β-adrenergic and muscarinic receptors on airway smooth muscle could be excluded (Kelly and Butcher, 1973; Shapiro et al., 1977; Lemberger, 1980). This conclusion was supported by the inability of THC to relax isolated rings of resting or precontracted human bronchioles (Orzelek-O'Neil et al., 1980a,b), suggesting a more proximal site of action in the lung (Cavero et al., 1972) or a central mechanism.

More recently, Calignano et al. (2000) reported that CB_1 receptors are present on axon terminals innervating airway smooth muscle, and anandamide inhibited capsaicin-induced bronchospasm and cough in guinea pigs in an SR141716-sensitive manner. They also documented calcium-induced biosynthesis of anandamide in lung tissue, suggesting that locally generated anandamide participates in the intrinsic control of airway responsiveness by inhibiting prejunctional acetylcholine release. Indeed, SR141716 treatment was found to enhance capsaicin-evoked bronchospasm and cough. Interestingly, when airway smooth muscle was completely relaxed by vagotomy and atropine treatment, anandamide caused dose-dependent bronchoconstriction, which

could be also prevented by CB_1 blockade. This effect was tentatively attributed to direct stimulation of putative cannabinoid receptors on the airway smooth muscle or a CB_1-mediated corelease of bronchoconstrictor neurotransmitters from nerve endings in the lung. In a follow-up study, presynaptic CB_1 receptors in the guinea pig lung were only found on noradrenergic terminals where their stimulation by WIN 55,212-2 inhibited norepinephrine release (Vizi et al., 2001), consistent with the lack of a mediatedCB_1-mediated effect on acetylcholine release in guinea pig trachea (Spicuzza et al., 2000). In contrast to the findings of Calignano et al. (2000), Stengel et al. (1998) reported that anandamide given either intravenously or in aerosol did not affect airway resistance in guinea pigs, but possessed modest anti-inflammatory properties. It should be noted, however, that in this study bronchoconstriction was induced by a calcium ionophore rather than capsaicin. In an in vitro study of guinea pig airway smooth muscle (Yoshihara et al., 2005), anandamide and palmitoylethanolamide inhibited contractions elicited by electrical field stimulation but not by neurokinin A, and also blocked capsaicin-capsaicin-induced release of substance P-like immunoreactivity. These effects were selectively inhibited by a CB_2 but not a CB_1 antagonist, or by maxi-K^+ channel blockers, suggesting that CB_2 agonists may have therapeutic value in asthma (Yoshihara et al., 2005). In a recent study, inhibition of anandamide transport potently suppressed capsaicin-induced cough in mice, suggesting that the anandamide transporter may be a target for peripherally acting antitussive medications (Kamei et al., 2006). Diverse effects of endocannabinoids and synthetic agonist have also been reported on respiratory function and pulmonary circulation both in vivo and in vitro (Schmid et al., 2003; Wahn et al., 2005).

Allergic asthma is currently viewed as a complex inflammatory disorder characterized by recruitment of eosinophils into the lung, mucus hypersecretion by goblet cells, elevated serum IgE, and airway hyperresponsiveness (reviewed in Wills-Karp, 1999). Given the well known anti-inflammatory effects of cannabinoids, these effects could also be of therapeutic value. Indeed, in a murine model of allergic airway disease induced by ovalbumin sensitization, pretreatment with cannabinol or THC blunted the increase in IL-2, IL-4, IL-5, and IL-13 mRNA expression and decreased mucus overproduction and serum IgE levels (Jan et al., 2003). Anti-inflammatory effects of WIN 55,212-2, THC, anandamide, and palmitoylethanolamide were also reported in a mouse model of LPS-induced pulmonary inflammation (Berdyshev et al., 1998).

In conclusion, the effects of cannabinoids on respiratory function are rather complex, and evidence for their therapeutic potential in asthma is equivocal. The possibility remains that novel, nonpsychoactive cannabinoid analogs with long-lasting anti-inflammatory activity turn out to be useful adjuncts in the treatment of allergic asthma.

E. Eye Disorders (Glaucoma and Retinopathy)

Glaucoma, the leading cause of irreversible blindness in the United States, is characterized by an increase in intraocular pressure and consequent damage to the optic nerve. Despite the multitude of effective medications that can be used to decrease ocular hypertension (e.g., cholinergic agonists, β- and α_2-adrenoceptor agonists, dopaminergic agonists, prostaglandins, and carbonic anhydrase inhibitors), some patients remain refractory to these drugs and may eventually become blind (reviewed in Alward, 1998; Crowston and Weinreb, 2005).

A decrease in intraocular pressure in a small number of healthy marijuana smokers was a serendipitous finding (Hepler and Frank, 1971), subsequently confirmed in a placebo-controlled, double-blind study of healthy volunteers who smoked either natural marijuana of known THC content or ingested synthetic THC (Hepler et al., 1972). THC or marijuana decreased intraocular pressure whether administered orally, topically, or intravenously, with no major tolerance to their effect reported (Shapiro, 1974; Purnell and Gregg, 1975; Cuendet et al., 1976; Hepler et al., 1976; Brown et al., 1977; Merritt et al., 1980, 1981a,b). Most of these studies also reported various systemic side effects, such as hypotension, tachycardia, euphoria, and dysphoria, as well as other ocular effects, such as changes in pupil size, decreased tear production, and conjunctival hyperemia. Endocannabinoids and synthetic cannabinoid ligands have also been reported to reduce intraocular pressure when given topically or systemically, both in animals and humans (Shapiro, 1974; ElSohly et al., 1981, 1984; Colasanti et al., 1984a,b,c; Pate et al., 1995; Porcella et al., 1998; Buchwald et al., 2002; Laine et al., 2002a,b; reviewed in Jarvinen et al., 2002; Chien et al., 2003; Tomida et al., 2004).

Early investigations into the mechanisms of the intraocular pressure-lowering effect of marijuana and THC implicated the sympathetic and central nervous systems in this effect (Green and Pederson, 1973; Green and Podos, 1974; Green et al., 1977a,b). However, in subsequent studies, the effect of a unilateral topical application of cannabinoids was limited to the treated eye, pointing toward a local site of action (Colasanti et al., 1984a,b,c). Indeed, a CNS site of action could be ruled out by the lack of change in intraocular pressure upon intracerebroventricular or ventriculocisternal application of THC in rabbits (Liu and Dacus, 1987).

Multiple lines of evidence suggest that endocannabinoids and cannabinoid receptors, in particular CB_1, play an important role in the regulation of intraocular pressure, and topically applied cannabinoids and cannabinoid ligands may be of significant benefit in the treatment of glaucoma (reviewed in Jarvinen et al., 2002; Tomida et al., 2004). First, CB_1 receptors are expressed

in the rat ciliary body (Porcella et al., 1998), in human ciliary epithelium, ciliary muscle, ciliary body vessels, trabecular meshwork, Schlemm's canal, and retina (Straiker et al., 1999a; Porcella et al., 2000; Stamer et al., 2001), and the retina of a variety of species (Straiker et al., 1999b; Yazulla et al., 1999, 2000). Second, ocular CB_1 receptors are functionally active, as CB_1 receptor agonists (CP55,940 and WIN 55,212-2) applied topically lower intraocular pressure both in animals and humans, and their effect can be antagonized by SR141716 (Pate et al., 1998; Song and Slowey, 2000; Porcella et al., 2001; Chien et al., 2003; Stumpff et al., 2005; reviewed in Jarvinen et al., 2002). The CB_2 receptor agonist JWH-133 did not modify the intraocular pressure, suggesting that CB_2 receptors may play only a minor, if any, role (Laine et al., 2003). CB_1 receptor signaling is also operational in the ocular trabecular meshwork (Stumpff et al., 2005), and ciliary muscle (Lograno and Romano, 2004). Third, endocannabinoids are detectable in ocular tissues including the retina, ciliary body, and choroids plexus (Bisogno et al., 1999b; Straiker et al., 1999a,b; Stamer et al., 2001; Chen et al., 2005), and the levels of anandamide and especially 2-AG are significantly decreased in patients with glaucoma (Chen et al., 2005).

The cellular/molecular mechanisms responsible for the intraocular pressure-reducing effect of cannabinoids are not completely understood but may involve direct effects on ciliary processes such as vasodilation and reduction of capillary pressure and secretion and do not seem to be related to systemic reduction of arterial blood pressure (Green and Pederson, 1973; Korczyn, 1980). Cannabinoids may also inhibit calcium influx through presynaptic ion channels, thereby reducing norepinephrine release in the ciliary body, resulting in decreased aqueous humor production (Sugrue, 1997). In addition, cannabinoids may improve the uveoscleral outflow by dilating blood vessels of the anterior uvea (Porcella et al., 1998), most likely by induction of several outflow-facilitating mediators (Rosch et al., 2006). Some evidence implicates prostanoids in the intraocular pressure-reducing effect of certain cannabinoids and anandamide (Pate et al., 1996; Green et al., 2001; Rosch et al., 2006).

Endocannabinoids as well as functional CB_1 receptors are present in the retina (Bisogno et al., 1999b; Straiker et al., 1999a,b; Fan and Yazulla, 2003; Savinainen and Laitinen, 2004). Cannabinoids exert neuroprotective effects against retinal neurotoxicity (El-Remessy et al., 2003), and cannabidiol helps to preserve the blood-retinal barrier in experimental diabetes (El-Remessy et al., 2006). These effects could suggest their usefulness in various retinopathies. Unlike CB_1 receptors, CB_2 receptors were undetectable in human retina, although they were found in the rat retina (Lu et al., 2000; Porcella et al., 2000).

Taken together, these findings indicate that cannabinoids may have great potential in the treatment of glaucoma, if the difficulty in formulating a stable and effective topical preparation and the problem of systemic side effects are conquered. Because of their well known neuroprotective, anti-inflammatory, and antiangiogenic effects, cannabinoid analogs may also be considered for the treatment of inflammatory and degenerative eye disorders and diabetic retinopathy.

F. Cancer

The palliative effects of cannabinoids in cancer patients are well known and may include appetite stimulation, inhibition of nausea and emesis associated with chemo- or radiotherapy, pain relief, mood elevation, and relief from insomnia (reviewed in Walsh et al., 2003; Hall et al., 2005) (Table 1). Δ^9-THC (dronabinol, Marinol) and its synthetic derivative nabilone have been approved by the U.S. Food and Drug Administration to control nausea in cancer patients undergoing chemotherapy and to stimulate appetite in patients with AIDS (Walsh et al., 2003; Hall et al., 2005).

Numerous recent studies have suggested that cannabinoids might directly inhibit cancer growth (reviewed in Parolaro et al., 2002; Guzmán et al., 2002; Guzmán, 2003; Jones and Howl, 2003; Velasco et al., 2004; Patsos et al., 2005). The proposed mechanisms are complex and may involve induction of apoptosis in tumor cells, antiproliferative action, and an antimetastatic effect through inhibition of angiogenesis and tumor cell migration (reviewed in Bifulco and Di Marzo, 2002; Parolaro et al., 2002; Guzmán et al., 2002; Guzmán, 2003; Jones and Howl, 2003; Velasco et al., 2004; Patsos et al., 2005).

Various cannabinoids, including cannabidiol, anandamide, and 2-AG, and endocannabinoid transport inhibitors have been shown to induce apoptotic cell death and to inhibit proliferation and migration in numerous murine and human tumor cell lines including glioma (C6, U87, U373, and H4), oligodendroglioma (Gos3), glioblastoma multiforme, astrocytoma (U373-MG, U87MG, and human grade IV astrocytoma), neuroblastoma (N18 TG2 and CHP100), pheochromocytoma (PC12), breast cancer (MCF-7, EFM-19, T47D, TSA-E1, and MDA-MB-231), prostate cancer (LNCaP, DU145, and PC3), colon carcinoma (SW 480), uterine cervix carcinoma (CxCa), thyroid cancer (KiMol), leukemia (CEM, HEL-92, HL60, and Jurkat cell lines), and lymphoid tumors (EL-4 and P815) tumor cells via CB_1/CB_2- and VR_1 receptor-dependent or independent (e.g., cyclooxygenase) mechanisms (De Petrocellis et al., 1998; Sánchez et al., 1998, 2003; Jacobsson et al., 2000; Maccarrone et al., 2000b; Sarker et al., 2000; McKallip et al., 2002a,b; Fowler et al., 2003; Jonsson et al., 2003; Mimeault et al., 2003; Bifulco et al., 2004; Contassot et al., 2004a,b; Hinz et al., 2004; Joseph et al., 2004; Kogan et al., 2004; Massi et al., 2004; Nithipatikom et al., 2004; Allister et al., 2005; Ellert-Miklaszewska et al., 2005; Herrera et al., 2005, 2006; Lombard et al., 2005; Powles et al., 2005; Sarfaraz et al., 2005; Vaccani et al., 2005; Carracedo et al., 2006;

Grimaldi et al., 2006; Ligresti et al., 2006b). More importantly, systemic or local treatment with cannabinoids inhibited the growth of various types of tumor or tumor cell xenografts in vivo, including lung carcinoma (Munson et al., 1975), glioma (Galve-Roperh et al., 2000; Sánchez et al., 2001a; Massi et al., 2004), thyroid epithelioma (Bifulco et al., 2001), lymphoma (McKallip et al., 2002a), and skin carcinoma (Casanova et al., 2003) in mice.

The proapoptotic effect of cannabinoids in tumor cells is complex and may involve increased synthesis of the proapoptotic sphingolipid ceramide (Galve-Roperh et al., 2000; Gómez del Pulgar et al., 2002a,b), ceramide-dependent up-regulation of the stress protein p8 and several downstream stress-related genes expressed in the endoplasmic reticulum (ATF-4, CHOP, and TRB3; Carracedo et al., 2006), prolonged activation of the Raf-1/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase signaling cascade (Galve-Roperh et al., 2000), and inhibition of Akt (Gómez del Pulgar et al., 2000; Ellert-Miklaszewska et al., 2005), c-Jun NH_2-terminal kinase and p38 mitogen-activated protein kinase (Galve-Roperh et al., 2000; Sarker et al., 2003; Hinz et al., 2004; Powles et al., 2005). As mentioned above, cannabinoids also inhibit the proliferation of various tumor cells, possibly through inhibition of adenylyl cyclase and the cAMP/protein kinase A pathway (Melck et al., 1999), induction of the cyclin-dependent kinase inhibitor $p27^{kip1}$ (Portella et al., 2003), a decrease in epidermal growth factor receptor expression and/or the attenuation of epidermal growth factor receptor tyrosine kinase activity (Casanova et al., 2003; Mimeault et al., 2003), and a decrease in the activity and/or expression of nerve growth factor or vascular endothelial growth factor tyrosine kinase receptors and prolactin (De Petrocellis et al., 1998; Melck et al., 2000; Portella et al., 2003). In addition to their proapoptotic and antiproliferative effects in tumor cells, cannabinoids also inhibit the expression of proangiogenic mediators or their receptors (e.g., vascular endothelial growth factor) and reduce vascular hyperplasia and cell migration, which play crucial roles in tumor growth and metastasis formation (Blázquez 2004;et al., 2003, 2004; Casanova et al., 2003; Portella et al., 2003).

In sharp contrast to the above, Hart et al. (2004) have demonstrated that treatment of lung cancer (NCI-H292), squamous cell carcinoma (SCC-9), bladder carcinoma (5637), glioblastoma (U373-MG), astrocytoma (1321N1), and kidney cancer (A498) cells with nanomolar concentrations of cannabinoids such as THC, anandamide, HU-210, and WIN 55,212-2 leads to rapid epidermal growth factor receptor- and metalloprotease-dependent cancer cell proliferation. However, the same study also documented that at micromolar concentrations cannabinoids induced cancer cell apoptosis, in agreement with previous reports (Hart et al., 2004). These results highlight the bimodal action of cannabinoids on cancer cell growth, with low concentrations being proproliferative and high concentrations having antiproliferative effects.

The key role of the immune system in controlling the development of cancers is supported by findings that immunosuppressed individuals are at increased risk for developing cancer. For example, there is increased incidence of non-Hodgkin's lymphoma, Burkitt's lymphoma, Kaposi's sarcoma, and cervical cancer in AIDS patients and increased susceptibility to various lymphomas and solid tumors after organ transplantation (Bhatia et al., 2001; Scadden, 2003; Abu-Elmagd et al., 2004; Oruc et al., 2004). This concept is particularly important, because cannabinoids have well-known immunosuppressant effects (reviewed in Klein, 2005), which may compromise antitumor immune responses. Indeed, THC enhances breast and lung cancer growth and metastasis by suppressing CB_2 receptor-mediated antitumor immune responses (Zhu et al., 2000; McKallip et al., 2005) and can also lead to increased susceptibility to infections with various pathogens such as herpes simplex virus, *Legionella pneumophila,* and Fried leukemia virus (Morahan et al., 1979; Cabral et al., 1986; Specter et al., 1991; Klein et al., 2000b).

Epidemiological studies investigating the relationship of cannabis smoking and various forms of cancer have yielded inconsistent results, thus failing to resolve the conflicting findings in animal models of cancer or in cancer cell lines (Taylor, 1988; Caplan and Brigham, 1990; Kuijten et al., 1992; Grufferman et al., 1993; Sidney et al., 1997; Barsky et al., 1998; Zhang et al., 1999; Efird et al., 2004; Llewellyn et al., 2004; Rosenblatt et al., 2004; reviewed in Hall et al., 2005). The variability of the effects of cannabinoids in different tumor models may be related to the differential expression of CB_1 and CB_2 receptors. Thus, cannabinoids may be effective in killing tumors that abundantly express cannabinoid receptors, such as gliomas, but may increase the growth and metastasis of other types of tumors, such as breast cancer, with no or low expression of cannabinoid receptors, due to the suppression of the antitumor immune response (McKallip et al., 2005). Nevertheless, the majority of the findings to date are encouraging and suggest that cannabinoids may be useful not only as palliative therapy but also because of their ability to inhibit tumor growth and metastasis.

G. Gastrointestinal and Liver Disorders

Cannabis has been used empirically for centuries to stimulate appetite and decrease emesis and diarrhea. Recent evidence indicates that the endocannabinoid system plays an important role in the control of gastrointestinal motility and secretion both under physiological conditions and in various gastrointestinal disorders (reviewed in Pertwee, 2001; Pinto et al., 2002a,b; Di Carlo and Izzo, 2003; Coutts and Izzo, 2004; Duncan et al.,

2005; Massa et al., 2005). Unexpectedly, recent data also implicate endocannabinoids and their receptors in several aspects of acute and chronic liver disease, including hemodynamic changes, modulation of inflammatory processes, fibrosis, and altered brain function (reviewed in Gabbay et al., 2005; Jimenez, 2005).

Numerous studies using autoradiography, immunohistochemistry, and/or reverse transcription-polymerase chain reaction demonstrated colocalization of CB_1 receptors with cholinergic neurons across the enteric nervous system, including sensory and interneuronal as well as motoneural cell bodies of the myenteric plexus, in mice (Mascolo et al., 2002; Pinto et al., 2002a,b; Casu et al., 2003; Izzo et al., 2003; Storr et al., 2004), rats (Adami et al., 2002; Coutts et al., 2002; Storr et al., 2002; Burdyga et al., 2004), guinea-pigs (Coutts et al., 2002; MacNaughton et al., 2004), and pigs (Kulkarni-Narla and Brown, 2000). CB_1 receptors are also colocalized with neuropeptide Y and vasoactive intestinal peptide in a small population of submucous plexus neurons (Kulkarni-Narla and Brown, 2000; Coutts et al., 2002). CB_1 receptor immunoreactivity was evident in normal human colonic epithelium, smooth muscle, and the submucosal myenteric plexus (Wright et al., 2005). Both CB_1 and CB_2 receptors were found on plasma cells in the lamina propria, whereas only CB_2 were detectable on macrophages (Wright et al., 2005). Endocannabinoids are also present in the gastrointestinal tact. Indeed, 2-AG was originally isolated from gut tissue (Mechoulam et al., 1995), and the intestinal content of anandamide was found to be regulated by feeding status (Gomez et al., 2002).

Although in earlier studies CB_1 receptor expression was undetectable in the liver relative to the brain (Porcella et al., 2002), several recent studies revealed the presence of low levels of both CB_1 mRNA (Bátkai et al., 2001; Michalopoulos et al., 2003; Biecker et al., 2004; Engeli et al., 2005; Osei-Hyiaman et al., 2005b; Teixeira-Clerc et al., 2006) and CB_1 immunoreactivity (Osei-Hyiaman et al., 2005b) in whole liver or in various types of cells present in the liver, including hepatocytes (Michalopoulos et al., 2003; Osei-Hyiaman et al., 2005b), stellate cells (Siegmund et al., 2005; Teixeira-Clerc et al., 2006), and vascular endothelial cells (Bátkai et al., 2001). CB_2 receptor mRNA was also detected in cirrhotic but not in normal liver tissue (Julien et al., 2005). Endocannabinoids are detectable in the liver or liver cells both in animals and humans at levels similar to those in the brain and play an important role under various physiological and pathophysiological conditions (Cravatt et al., 2004; Kurabayashi et al., 2005; Osei-Hyiaman et al., 2005b) (see also section III.A.3.).

A functional role for endocannabinoids and CB_1 receptors in the gastrointestinal tract is supported by pharmacological studies demonstrating that anandamide and various CB_1 agonists (WIN 55,212-2, CP55,940, and ACEA) but not the CB_2-selective agonists JWH-133 inhibit gastrointestinal motility in rodents in vivo and in isolated ileum and colon from both experimental animals and humans (Shook and Burks, 1989; Pertwee et al., 1995, 1996; Coutts and Pertwee, 1997; McCallum et al., 1999; Mancinelli et al., 2001; Mang et al., 2001; Landi et al., 2002; Manara et al., 2002; Hinds et al., 2006). A similar role for endogenous substrates of FAAH is suggested by recent in vivo findings in mice, documenting inhibition of intestinal motility by the FAAH inhibitors *N*-arachidonoylserotonin and palmitoylisopropylamide and by the FAAH substrates palmitoylethanolamide, oleamide, and oleoylethanolamide in wild-type but not in FAAH knockout mice (Capasso et al., 2005). Furthermore, the effect of *N*-arachidonoylserotonin was reduced either by the CB_1 receptor antagonist SR141716 or by CB_1 deficiency, but not by the $TRPV_1$ receptor antagonist 5′-iodoresiniferatoxin (Capasso et al., 2005). Interestingly, in clinical trials using rimonabant for nicotine cessation or for the treatment of obesity, diarrhea was 2 to 2.4 times more frequent among subjects treated with the drug than with placebo, suggesting accelerated transit and/or enhanced secretion caused by CB_1 blockade (Fernandez and Allison, 2004; Van Gaal et al., 2005). This and some of the above experimental reports suggest the existence of an inhibitory endocannabinoid tone in the gastrointestinal tract. Multiple mechanisms, including reduction of acetylcholine release from enteric nerves, inhibition of nonadrenergic/noncholinergic excitatory transmission, activation of apamin-sensitive K^+ channels, and modulation of adenosine release have been proposed to explain the CB_1-mediated reduction in enteric contractility and peristalsis (reviewed in Coutts and Izzo, 2004).

Activation of both CB_1 and CB_2 receptors may decrease the pathologically increased intestinal motility elicited by an inflammatory stimulus. In a mouse model of croton oil-induced intestinal inflammation, the increased efficacy of cannabinoids in inhibiting intestinal motility was attributed to up-regulation of intestinal CB_1 receptors (Izzo et al., 2001a,b). Conversely, the accelerated gastrointestinal transit induced by bacterial endotoxin in rats could be inhibited by CB_2 but not CB_1 receptor agonists (Mathison et al., 2004). Interestingly, intestinal hypomotility in a mouse model of paralytic ileus has been linked, at least in part, to the enhancement of anandamide levels and CB_1 expression in the gut, and it could be attenuated by CB_1 receptor antagonists (Mascolo et al., 2002). Additionally, there is evidence that CB_1 receptors are involved in the regulation of the lower esophageal sphincter, and CB_1 activation might be beneficial in gastroesophageal reflux disease (reviewed in Coutts and Izzo, 2004; Massa et al., 2005).

The endocannabinoid system has also been implicated in the regulation of gastric acid and intestinal secretions. At high doses, THC decreased histamine-induced gastric acid secretion in isolated stomach preparations

(Rivas-V and Garcia, 1980) and in pylorus-ligated rats (Sofia et al., 1978). Pentagastrin-induced gastric acid secretion was also inhibited by HU-210 and WIN 55,212-2, an effect that could be prevented by CB_1 blockade (Coruzzi et al., 1999; Adami et al., 2002). These studies suggest a role for CB_1 receptors located on preganglionic and postganglionic cholinergic pathways in the regulation of gastric acid secretion. The therapeutic relevance of this regulatory mechanism was highlighted by the CB_1 receptor-mediated antiulcer activity of ACEA or WIN 55,212-2 treatment in a rat model of aspirin- and cold/restraint stress-induced gastric ulcers (Germano et al., 2001; Rutkowska and Fereniec-Goltbiewska, 2006). WIN 55,212-2 also reduced intestinal secretions evoked by electrical field stimulation or capsaicin (MacNaughton et al., 2004). Anandamide, the anandamide transport inhibitor VDM11, and the CB_1 agonist ACEA all inhibited intestinal secretion and fluid accumulation in mice treated with cholera toxin, whereas SR141716 exerted opposite effects (Izzo et al., 2003). The ability of cannabinoids to inhibit gastrointestinal motility and secretion coupled with their anti-inflammatory properties strongly suggests that the modulation of this system could offer significant benefits in the treatment of various gastrointestinal pathological conditions, including inflammatory bowel disease (see below).

1. Inflammatory Bowel Disease. Idiopathic inflammatory bowel disease (IBD) includes ulcerative colitis and Crohn's disease, and is characterized by intestinal inflammation presumably of autoimmune origin and a chronic relapsing course associated with local and systemic complications and affects >1 million people in the United States (Loftus, 2004). Although the etiology of IBD remains unclear, it may involve complex genetic, environmental, and immunological interactions. The most common symptoms of IBD are abdominal pain and diarrhea, which eventually lead to malabsorption and malnutrition, and in approximately half of patients surgery is eventually required to remove the affected bowel segment. Despite recent therapeutic advances, patients with IBD are often unresponsive to available treatment options.

As discussed above, the endocannabinoid system plays an important role in the control of gastrointestinal motility and secretion. Studies using animal models of IBD have suggested that targeting the endocannabinoid system may offer significant benefits in the treatment of IBD. Several studies have indicated that chemically induced intestinal inflammation is associated with the up-regulation of intestinal CB_1 receptors, which may represent a compensatory, protective mechanism. For example, in croton oil-treated mice, the ability of CB_1 agonists to inhibit intestinal motility is increased compared with that in control animals (Izzo et al., 2001a). More importantly, the anandamide transport inhibitor VDM11 was also shown to inhibit gastrointestinal motility and secretions in cholera toxin-treated mice, which implicates endocannabinoids in this action and holds out the promise of a nonpsychoactive form of treatment (Izzo et al., 2003). In a mouse model of colitis induced by 2,4-dinitrobenzene sulfonic acid and dextrane sulfate, Massa et al. (2004) have confirmed the up-regulation of CB_1 receptors in experimental colitis. Furthermore, they demonstrated that the inflammation was more severe in mice deficient in CB_1 receptors than in wild-type mice, whereas genetic ablation of FAAH resulted in protection against this chemically induced colitis (Massa et al., 2004). In a recent study, the anandamide reuptake inhibitor VDM11 afforded protection against colitis in mice, and elevated anandamide levels have been measured in biopsy material from patients with ulcerative colitis (D'Argenio et al., 2006). These findings strongly support the natural protective role of the endocannabinoid system in this form of experimental IBD. In contrast, Croci et al. (2003) have reported a CB_1 receptor-independent protective effect of SR141716 against indomethacin-induced inflammation and ulcer formation in the small intestine of rats. Elevated levels of anandamide and desensitization of the presynaptic neural CB_1 receptor found in colonic longitudinal muscle strips from patients undergoing surgery for complicated diverticulitis suggest that the endocannabinoid system may be also involved in the pathophysiology of this frequent complication of colitis and/or colon cancer (Guagnini et al., 2006).

Taken together, most of the above studies suggest that the endocannabinoid system in the gut is activated during inflammation, and endogenous anandamide may counteract inflammation (Kunos and Pacher, 2004) (Fig. 6). The findings of Massa et al. (2004) and D'Argenio et al. (2006) also suggest that inhibitors of FAAH or anandamide reuptake may amplify the natural protective action of endogenous anandamide, which warrants further studies to test such inhibitors in the treatment of experimental and, ultimately, human IBD (Kunos and Pacher, 2004). Future studies should further explore the mechanisms of the anti-inflammatory effects of cannabinoids and the potential role of CB_2 receptors as therapeutic targets (Mathison et al., 2004; Wright et al., 2005).

2. Acute and Chronic Liver Disease (Hepatitis and Liver Cirrhosis). Endocannabinoids and CB_1 receptors have been implicated in the systemic and portal vasodilation and hypotension associated with chronic liver cirrhosis (Bátkai et al., 2001; Garcia et al., 2001; Ros et al., 2002). These studies demonstrated that CB_1 receptor blockade with SR141716 reversed the hypotension and low peripheral resistance and decreased the elevated mesenteric blood flow and portal pressure in rats with biliary and carbon tetrachloride-induced cirrhosis, whereas these hemodynamic parameters were unaffected by SR141716 in noncirrhotic control subjects

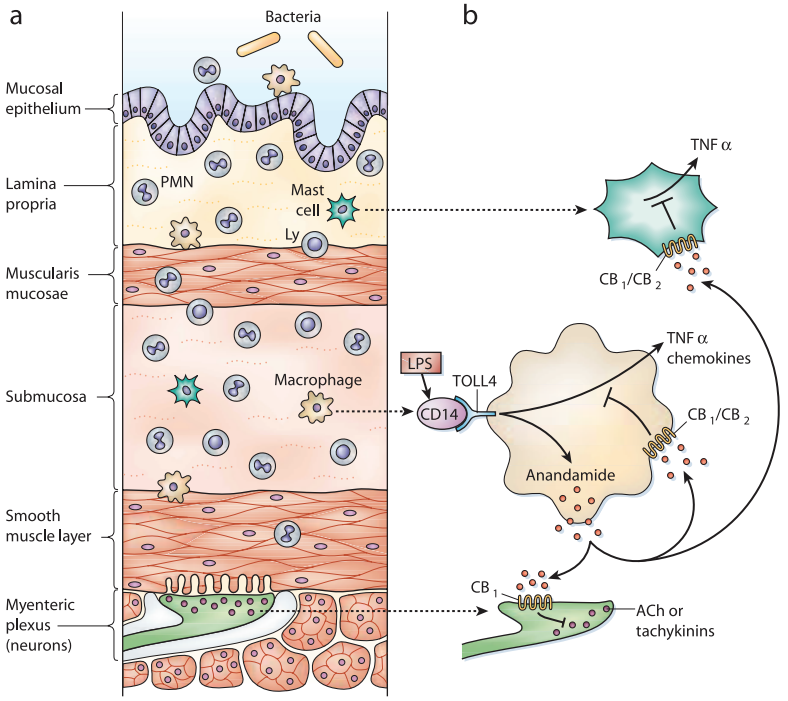


FIG. 6. Cellular source and proposed targets of anti-inflammatory endocannabinoids in inflammatory bowel disease. a, cross-section of inflamed bowel with leukocyte infiltration [polymorphonuclear leukocytes (PNM), lymphocytes (Ly), macrophages, and mast cells]. b, in macrophages, LPS induces the production of TNF-α and chemokines (such as MIP-2macrophage inflammatory protein-2 and CXCL-8) as well as anandamide. Anandamide is released to act as an autocrine mediator to inhibit TNF-α and chemokine production via CB_1 or CB_2 receptors or both. Activation of CB_1 and CB_2 receptors may similarly inhibit TNF-α production in mast cells, with these effects resulting in decreased leukocyte infiltration and inflammation. Paracrine activation of CB_1 receptors on extrinsic and intrinsic enteric neurons inhibits acetylcholine (ACh) and tachykinin release, respectively, resulting in inhibition of gut motility. These effects are amplified by treatment with a FAAH inhibitor, which prevents the breakdown of anandamide. Reproduced with permission from Kunos and Pacher (2004) *Nat Med* **10:**678–679. © Nature Publishing Group.

(Bátkai et al., 2001; Ros et al., 2002). These findings suggested an increased endocannabinoid tone in cirrhosis, which could be attributed to both an up-regulation of CB_1 receptors in hepatic vascular endothelial cells and an increased production of anandamide by circulating monocytes (Bátkai et al., 2001). Increased expression of CB_1 receptors was also reported in whole liver from bile duct-ligated mice (Biecker et al., 2004). This increase was greater when bile duct ligation was performed in NO synthase-3 knockout compared with wild-type mice, which may account for the similar level of portal hypertension in the two strains despite the much higher systemic blood pressure in the knockout mice (Biecker et al., 2004). Increased anandamide-induced vasorelaxation mediated by CB_1 and $TRPV_1$ receptors was also reported in mesenteric arteries isolated from cirrhotic compared with control rats (Domenicali et al., 2005). The increase in anandamide in monocytes from cirrhotic rats or humans is functionally important, as these cells elicit SR141716-sensitive hypotension when injected into normal recipient rats (Bátkai et al., 2001; Ros et al., 2002). Plasma endotoxin levels progressively increase as liver function deteriorates in cirrhosis (Lumsden et al., 1988; Chan et al., 1997), and this effect is probably responsible for the elevated endocannabinoid production in plasma monocytes and platelets of cirrhotic animals and patients (Bátkai et al., 2001; Ros et al., 2002; Liu et al., 2003; Fernandez-Rodriguez et al., 2004). There is also recent experimental evidence implicating increased signaling through myocardial CB_1 receptors in the pathogenesis of cirrhotic cardiomyopathy (Gaskari et al., 2005; Pacher et al., 2005c).

Beyond the vasculopathy of end-stage cirrhosis, the endocannabinoid system may also be involved in the pathogenesis of liver fibrosis. Siegmund et al. (2005) have recently reported that anandamide exerts antifibrogenic effects in vitro by inhibiting activated hepatic stellate cells at low micromolar concentrations and by inducing their necrosis at higher concentrations, via $CB_{1/2}$- and $TRPV_1$-independent mechanism(s). In a study by Julien et al. (2005), the liver fibrosis induced by carbon tetrachloride was more severe in CB_2 knockout mice compared with their wild-type littermates. Also, the expression of CB_2 receptors was found to be strongly induced in liver biopsy specimens from patients with active cirrhosis of various etiologies, particularly in nonparenchymal cells located within and at the edge of fibrous septa (Julien et al., 2005). Furthermore, CB_2 receptor activation triggered growth inhibition and apoptosis in myofibroblasts and in activated hepatic stellate cells, highlighting the antifibrogenic role of CB_2 receptors during chronic liver injury (Julien et al., 2005). However, chronic marijuana use has been associated with hepatotoxicity rather than hepatoprotection as ex-

pected from the above results (Borini et al., 2004), and results of a recent epidemiological study indicate that daily marijuana smoking is a risk factor for progression of fibrosis among people with chronic hepatitis C infection (Hezode et al., 2005). This finding has triggered an investigation into the possible pro-fibrogenic role of CB_1 receptor activation, which is supported by the results of a preliminary study showing that the progression of experimental liver fibrosis induced by carbon tetrachloride is slower in mice with genetic ablation of CB_1 receptors or treated with CB_1 receptor antagonist SR141716 (Teixeira-Clerc et al., 2006). These latter findings suggest a broader role of CB_1 receptors in the pathogenesis of cirrhosis and forecast additional potential benefits from the therapeutic use of a CB_1 antagonist in chronic liver disease.

In contrast to the hepatotoxicity associated with chronic marijuana use, a synthetic, nonpsychotropic cannabinoid derivative (PRS-211,092) was reported to inhibit acute hepatitis induced by concanavalin A via negative cytokine regulation in mice (Lavon et al., 2003). Interestingly, in animal models of acute hepatic failure-induced encephalopathy, both 2-AG and SR141716 have been reported to exert beneficial effects on neurological and cognitive function (Gabbay et al., 2005; Avraham et al., 2006). Cannabinoids may also be beneficial in intractable cholestatic pruritus (Neff et al., 2002), which is associated with severe forms of liver disease, presumably by increasing the nociceptive threshold (Gingold and Bergasa, 2003).

Collectively, the studies discussed in this section highlight the potential regulatory role of the endocannabinoid system in a variety of gastrointestinal and liver disorders, opening new avenues for their pharmacotherapy. It appears that CB_1 agonists and perhaps FAAH antagonists might be beneficial in reducing increased gastrointestinal motility, bowel inflammation, and associated diarrhea, whereas CB_1 antagonists could be used in the treatment of constipation. In chronic liver cirrhosis, CB_1 antagonists may not only attenuate or reverse the adverse hemodynamic consequences of cirrhosis, thus extending life until a suitable liver becomes available for transplantation, but also could have additional benefits by slowing the progression of fibrosis and the neurological decline associated with hepatic encephalopathy. Selective CB_2 receptor agonists might also be expected to protect against progression of liver fibrosis and perhaps against the chronic inflammation associated with IBD.

H. Musculoskeletal Disorders

1. Arthritis. Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory autoimmune disease affecting ~0.8% of adults worldwide. RA is more common in women, and it leads to joint destruction, deformity, loss of function, chronic pain, and reduced quality of life. When unchecked, it leads to substantial disability and premature death (O'Dell, 2004). Current medications used to treat rheumatoid arthritis are divided into three main classes: nonsteroidal anti-inflammatory drugs, corticosteroids, and disease-modifying antirheumatic drugs such as methotrexate (O'Dell, 2004). A better understanding of the cytokine networks that are responsible for the ongoing inflammatory response in RA has led to the successful use of novel therapies that target TNF-α and IL-1.

The immunosuppressant and anti-inflammatory properties of cannabinoids are highly relevant for RA and other autoimmune disorders (e.g., systemic lupus erythematosus, autoimmune vasculitis, Sjögren's syndrome, and ankylosing spondylitis). Indeed, ajulemic acid (THC-11-oic acid, CT-3, IP-751), a potent analog of the acid metabolites of THC (Burstein, 2000, 2005) and cannabidiol have been shown to have analgesic, anti-inflammatory, and immunosuppressive effects in animal models of arthritis (Zurier et al., 1998; Dajani et al., 1999; Malfait et al., 2000). Chronic administration of ajulemic acid attenuated joint inflammation in a murine model of adjuvant-induced arthritis and suppressed prostaglandin production in vitro to a greater extent than the potent nonsteroidal anti-inflammatory drug, indomethacin (Zurier et al., 1998). In another study, ajulemic acid caused less gastrointestinal ulcerations and was more effective in reducing adjuvant-induced arthritis than common nonsteroidal anti-inflammatory agents (Dajani et al., 1999). As discussed earlier in this review, ajulemic acid is a high-affinity agonist for human cannabinoid receptors and has CB_1-mediated, potent antihyperalgesic activity in models of chronic neuropathic and inflammatory pain in the rat (Dyson et al., 2005). Ajulemic acid also induces apoptosis in human T lymphocytes (Bidinger et al., 2003) and suppresses IL-1β production in human monocytes (Zurier et al., 2003), which could contribute to its therapeutic effects in RA and other inflammatory disorders. Treatment with cannabidiol or its more potent dimethylheptyl derivative (HU-320) reduced an LPS-induced increase in serum TNF-α and immune function and effectively blocked the progression of collagen-induced arthritis in mice (Malfait et al., 2000; Sumariwalla et al., 2004). Other studies described the antinociceptive effects of anandamide and THC in rats with arthritis (Sofia et al., 1973; Smith et al., 1998; Cox and Welch, 2004). Mbvundula et al. (2005, 2006) have recently reported that WIN 55,212-2 and HU-210 inhibited IL-1-stimulated NO production in bovine articular chondrocytes, in contrast to AM281 and AM630, which elicited an opposite effect. Anandamide, WIN 55212-2, and HU-210 also inhibited the release of sulfated glycosaminoglycans in bovine cartilage explants and IL-1a stimulated proteoglycan and collagen degradation (Mbvundula et al., 2005, 2006).

In a survey of 2969 people using cannabis for medicinal purposes, ~25% of subjects mentioned relief of ar-

thritis symptoms as the main reason for cannabis smoking, which was surpassed only by chronic pain, MS, and depression (Ware et al., 2003). Studies using cannabinoid-based extracts are also underway in patients with RA (Russo, 2006). The potential benefit of cannabinoids in fibromyalgia, a syndrome of widespread musculoskeletal pain, nonrestorative sleep, disturbed mood, and fatigue of unknown etiology, has also been reviewed (Russo, 2004).

2. Osteoporosis. Osteoporosis is a skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone, leading to increased susceptibility to bone fractures. The associated fractures and the subsequent morbidity and mortality make osteoporosis an enormous public health concern. Osteoporosis is no longer considered an age-related disease, as it is increasingly recognized in children. Osteoporosis is thought to be a polygenic disorder, with vulnerability determined by multiple genes and environmental risk factors. It currently affects up to one in three women and 1 in 12 men worldwide (Keen, 2003). Treatment options include general measures on lifestyle, calcium and vitamin D supplements, hormone therapy, raloxifene, and bisphosphonates.

Cannabinoid receptors were first implicated in the regulation of bone mass by Karsak et al. (2004), who found that CB_2 knockout mice had markedly accelerated age-related trabecular bone loss and cortical expansion accompanied by increased activity of trabecular osteoblasts, increased numbers of osteoclasts, and decreased numbers of diaphyseal osteoblast precursors (Ofek et al., 2006). CB_2 receptors were expressed in osteoblasts, osteocytes, and osteoclasts. The selective CB_2 agonist HU-308, but not the CB_1 agonist noladine ether, attenuated ovariectomy-induced bone loss and markedly stimulated cortical thickness through the suppression of osteoclast number and stimulation of endocortical bone formation (Ofek et al., 2006). Furthermore, HU-308 dose dependently increased the number and activity of endocortical osteoblasts and restrained trabecular osteoclastogenesis by inhibiting proliferation of osteoclast precursors (Ofek et al., 2006). These results, coupled with CB_2 but not CB_1 receptor mRNA expression during osteoblastic differentiation, suggested a role for CB_2 receptors in bone remodeling. Such a role of CB_2 but not CB_1 receptors is also supported by a recent genetic association study in human samples of postmenopausal osteoporosis patients and matched female control subjects (Karsak et al., 2005).

In contrast, Idris et al. (2005) have recently reported that CB_1 receptor knockout mice or mice treated with antagonists of either CB_1 or CB_2 receptors were protected from ovariectomy-induced bone loss. Furthermore, cannabinoid antagonists promoted osteoclast apoptosis, inhibited osteoclast activity, and decreased the production of several osteoclast survival factors in vitro, suggesting that cannabinoid antagonists may be beneficial in the treatment of osteoporosis. Although the reason for the discrepancy between the above studies is not clear; they suggest a role for the endocannabinoid system in the regulation of bone mass.

I. Endocannabinoids and Reproductive Functions

There is abundant evidence that the endocannabinoid system is involved in reproductive functions in both males and females and in both animals and humans, as discussed in more detail in recent reviews (Fride, 2004; Park et al., 2004; Schuel and Burkman, 2005; Tranguch et al., 2005; Wang et al., 2006). In males, marijuana, synthetic cannabinoids, and anandamide adversely affect the fertilizing capacity of sperm, which express functional CB_1 receptors (Rossato et al., 2005; Schuel and Burkman, 2005; Whan et al., 2006). On the other hand, there is preclinical evidence to suggest that blockade of CB_1 may be useful in the treatment of erectile dysfunction (Melis et al., 2004b, 2006).

High levels of functional CB_1 receptor, anandamide, and FAAH are present in the preimplantation embryo and/or in the uterus (Das et al., 1995; Paria et al., 1995, 2001; Schmid et al., 1997; Park et al., 2003; Guo et al., 2005). Anandamide synthesized in the uterus exerts dose- and stage-specific effects on embryo development and implantation. A temporary reduction of anandamide levels is essential for embryo implantation, and higher anandamide levels are associated with uterine nonreceptivity and impairment of blastocyst formation, zona hatching, and trophoblast outgrowth via CB_1 receptors (Das et al., 1995; Paria et al., 1995, 2001, 2002; Schmid et al., 1997; Wang et al., 1999; Guo et al., 2005). Consequently, cannabinoids may retard the development of embryos, eventually leading to fetal loss and pregnancy failure (Bloch et al., 1978; Smith and Asch, 1987; Park et al., 2004). Anandamide levels in the uterus are regulated by FAAH activity (Paria et al., 1995, 1999; Schmid et al., 1997). Accordingly, pregnant women with low FAAH activity in lymphocytes were found to have an increased incidence of miscarriage (Maccarrone et al., 2000c), and low FAAH activity also correlated with failure to maintain pregnancy after in vitro fertilization (Maccarrone et al., 2002b). Finally, cannabinoids may also affect the levels of various hormones crucial for normal fertility and reproduction (Brown and Dobs, 2002; Park et al., 2004; Scorticati et al., 2004; Gammon et al., 2005). Although such findings may suggest the potential usefulness of CB_1 antagonists in the treatment of infertility problems, a note of caution is warranted because CB_1 knockout mice were reported to have impaired oviductal transport of embryos, leading to embryo retention. This suggests that treatment with CB_1 antagonists may facilitate ectopic pregnancy (Wang et al., 2004).

TABLE 1
Clinical trials with cannabinoid-related medications in human disease

Disease/Condition	Sample Size, Design, Target Symptoms	Compound (Dose)	Parameters Studied	Results	Adverse Effects	Reference
MS and SCI						
MS	Nine patients, DB, PL, spasticity	THC (5- and 10-mg single-dose p.o.)	EMG, clinical	Improved spasticity score (objective)	Minimal	Petro and Ellenberger (1981)
MS	Eight patients, SB, PL, tremor, ataxia	THC (5 mg/6 h max three doses p.o.)	Clinical	Improved coordination and sense of well being, decreased tremor (subjective)	Subjective "high" in all patients	Clifford (1983)
MS	13 patients, DB, PL, C, spasticity	THC (2.5–15 mg daily for 5 days p.o.)	Clinical, questionnaire	Reduced spasticity (subjective); objective function tests not improved	Common	Ungerleider et al. (1987)
MS	One patient, OL, spastic tetraparesis	Cigarette smoke marijuana (one cigarette)	Clinical, tremor recording, EMG	Reduced ataxia and spasticity (objective)	None	Meinck et al. (1989)
MS	10 patients, DB, C, spasticity	Cigarette smoke marijuana (one cigarette; 1.54% THC)	Dynamic posturography, objective balance	Impaired posture and balance	Subjective unpleasant "high" in all patients	Greenberg et al. (1994)
MS	One patient, DB, PL, C, spasticity	Nabilone (1 mg/2 days for 16 wk p.o.)	Visual analog scales	Improved painful muscle spasms, mood and well being (subjective); reduced frequency of nocturia	Mild sedation	Martyn et al. (1995)
MS and SCI	Two patients, OL, spasticity	THC (10 or 15 mg p.o. or rectal)	Clinical	Improved walking ability and passive mobility, reduced rigidity, slight pain relief	Temporal deterioration in ability to concentrate and in mood	Brenneisen et al. (1996)
MS	One patient, PL, nystagmus	Cigarette smoke marijuana (inhaled)	Eye movement recording	Reduced nystagmus amplitude and improved visual acuity	None	Schon et al. (1999)
MS	16 patients, DB, PL, C, spasticity	Plant extract of THC (2.5–5 mg b.i.d. for 4 wk p.o.)	Clinical, questionnaires, Ashworth score	No improvement in Ashworth scale, worsening global impression	41 adverse events in 16 patients during plant extract treatment	Killestein et al. (2002)
MS and SCI	24 patients, DB, PL, C, heterogeneous	Plant extract of THC and CBD 1:1 (2.5–120 mg/day for 2 wk sublingual)	Clinical, questionnaires	Improvement of bladder control, muscle spasms, and spasticity and pain relief (subjective) but no in Ashworth scale	4 dropouts due to adverse events	Wade et al. (2003)
MS	630 patients, DB, R, PL, spasticity	Cannabis extract (Cannador: 2.5 mg Δ^9-THC + 1.25 mg CBD/capsule; Marinol: THC max 25 mg/day for 15 wk p.o.)	Clinical, questionnaires Ashworth score, Rivermead Mobility Index	No change in the Ashworth score, but improvement in the patient-reported spasticity, pain, and sleep quality; unexpected reduction in hospital admission for relapse in the treatment groups; in 12-mo follow-up, THC improved muscle spasticity measured by the Ashworth scale and the Rivermead Mobility Index	Minimal, similar to placebo	Zajicek et al. (2003, 2004)
MS	57 patients, DB, R, PL, C, spasticity, various	Cannabis-based capsules (2.5 mg THC and 0.9 mg CBD; max dose 30 mg/day THC p.o.)	Self-report of spasm frequency and symptoms, Ashworth Scale, Rivermead Mobility Index, 10-m timed walk	Improved spasm frequency and mobility in the 37 patients who received at least 90% of their prescribed dose	Minor adverse events were slightly more frequent in treated group	Vaney et al. (2004)
MS	14 patients, DB, PL, tremor	Cannabis extract (Cannador: 2.5 mg Δ^9-THC + 1.25 mg CBD/capsule p.o. for 2 wk)	Tremor index, measured using a validated tremor rating scale	No effects on tremor	Minimal	Fox et al. (2004)

TABLE 1
Continued

Disease/Condition	Sample Size, Design, Target Symptoms	Compound (Dose)	Parameters Studied	Results	Adverse Effects	Reference
MS	57 patients, DB, R, PL, C, spasticity, various	Cannabis-based capsules (2.5 mg THC and 0.9 mg CBD; max dose 30 mg/day THC p.o.)	Self-report of spasm frequency and symptoms, Ashworth Scale, Rivermead Mobility Index, 10-m timed walk	Improved spasm frequency and mobility in the 37 patients who received at least 90% of their prescribed dose	Minor adverse events were slightly more frequent in treated group	Vaney et al. (2004)
MS	14 patients, DB, PL, tremor	Cannabis extract (Cannador: 2.5 mg Δ^9-THC + 1.25 mg CBD/capsule p.o. for 2 wk)	Tremor index, measured using a validated tremor rating scale	No effects on tremor	Minimal	Fox et al. (2004)
MS	160 patients, DB, PL, R, M, VAS score for each patient's most troublesome symptom	GW-1000 (Sativex) delivered by oromucosal spray (2.7 mg Δ^9-THC and 2.5 mg CBD at each actuation)	VAS score for each patient's most troublesome symptom, Ashworth Scale	No significant difference in the Ashworth scale, tremor, and pain at 6 wk between the Sativex and placebo groups; improved VAS scores for spasticity	Minimal	Wade et al. (2004)
Pain (see also MS above)						
Cancer	10 patients, P, non-R, non-DB, pain	THC (5, 10, 15, or 20 mg p.o.)	Cancer-associated pain	Superior to PL	Common at higher doses	Noyes et al. (1975a)
Cancer	34 patients, P, non-R, C, pain	THC (20 mg, codeine 120 mg p.o.)	Cancer-associated pain	Both superior to PL	Common with THC	Noyes et al. (1975b)
Cancer	45 patients, DB, C, P, pain	NIB (4 mg, codeine 50 mg, secobarbital 50 mg)	Cancer associated pain	NIB equal to codeine, superior to secobarbital and PL	Common	Staquet et al. (1978)
Dental extraction	10 patients, DB, R, PL	THC (0.022, 0044 mg, diazepam 0.157 mg/kg i.v.)	Surgical pain	THC superior to PL, inferior to diazepam	Not discussed	Raft et al. (1977)
FMF	One patient, DB, R, C	THC (50 mg daily p.o.)	Gastrointestinal pain	Superior to PL	Not discussed	Holdcroft et al. (1997)
MS	One patient, OL, pain	Nabilone (1 mg b.i.d. p.o.)	Questionnaire, various	Complete pain relief	None	Hamann and di Vadi (1999)
MS	66 patients, DB, R, PL, pain, sleep disturbances	Sativex delivered by oromucosal spray (2.7 mg Δ^9-THC and 2.5 mg CBD at each actuation)	Pain, sleep disturbances, numerical rating scale	Improved central neuropathic pain and sleep disturbances	Minimal	Rog et al. (2005)
Neuropathy of varying etiologies	21 patients, DB, R, C, PL, pain	Ajulemic acid (CT-3, IP-751: 4 or 10 mg p.o. two times daily)	Neuropathic pain, VAS	Significant reduction of chronic neuropathic pain	Minimal	Karst et al. (2003)
HIV	523 patients, cross-sectional questionnaire study	Cannabis	Questionnaire, various	In most patients who used cannabis to treat symptoms (143/523); reduction in muscle and neuropathic pain	Not discussed	Woolridge et al. (2005)

TABLE 1
Continued

Disease/Condition	Sample Size, Design, Target Symptoms	Compound (Dose)	Parameters Studied	Results	Adverse Effects	Reference
Anorexia-cachexia in patients with cancer, HIV, or AIDS						
Cancer	54 patients, R, DB, weight	THC (three doses of 0.1 mg/kg/day p.o.)	Appetite, weight	Improved appetite and increased weight	Dizziness, sedation, confusion	Regelson et al. (1976)
Cancer	19 patients, OL, non-R, weight	THC (three doses of 5 mg/day p.o.)	Appetite, weight	Improved appetite, trends for weight increase	Common, but well-tolerated	Nelson et al. (1994)
HIV/AIDS	10 patients, non-R, weight	THC (three doses of 2.5 mg/day p.o.)	Weight	Increased/stabilized weight	Mild	Gorter et al. (1992)
AIDS	139 patients, R, PL, weight	THC (two doses of 2.5 mg/day p.o.)	VAS for hunger, weight	Improved VAS for hunger but not weight	Mild	Beal et al. (1995)
AIDS	94 patients, non-R, OL, weight	THC (two doses of 2.5 mg/day p.o.)	VAS for hunger, weight	Improved VAS for hunger and weight (only for 1 month)	Sedation, psychosis, dysphoria	Beal et al. (1997)
AIDS	52 patients, weight	THC (two doses of 2.5 mg/day p.o. +/− Megace)	VAS for hunger, weight	Less effective than Megace	Anxiety, euphoria, psychosis, confusion	Timpone et al. (1997)
Chemotherapy-induced nausea and vomiting						
Chemotherapy-induced nausea and vomiting	Review of 30 randomized trials involving 1366 patients, nausea, vomiting	THC	Nausea, vomiting	Across all trials, cannabinoids were more effective than placebo	Various	Tramèr et al. (2001)
Traumatic brain injury						
Closed head injury	67 patients, R, DB, PL, phase II, M, neurological outcome	HU-211 (dexanabinol: 48 or 150 mg i.v.)	Intracranial, cerebral perfusion and blood pressure, Glasgow scale	Better intracranial pressure/cerebral perfusion pressure control, trends towards better neurological outcome	Similar in all groups, related to severe head trauma	Knoller et al. (2002)
Traumatic brain injury	861 patients, R, PL, phase III, M, neurological outcome	HU-211 (dexanabinol: 150 mg i.v.)	Extended Glasgow scale at 6 months	No improvement	Similar in all groups, related to severe head trauma	Maas et al. (2006)
Parkinson's disease, levodopa-induced dyskinesia						
Parkinson's disease	24 patients, R, DB, PL, motor disability	SR141716 (0.3 mg/kg p.o.); antagonists of NK_3 R (SR142801) and neurotensin receptors (SR48692)	Motor symptoms and levodopa-induced dyskinesias after a single dose of levodopa	No improvement in parkinsonian motor disability with any of drugs tested	Minimal	Mesnage et al. (2004)
Parkinson's disease	Seven patients, R, DB, PL, C, motor disability	Nabilone	Motor symptoms	Reduces levodopa-induced dyskinesia in PD	Minimal	Sieradzan et al. (2001)
Alzheimer's disease, dementia						
Alzheimer's disease, dementia	Six patients, OL, pilot, neuropsychiatric symptoms	Dronabinol (2.5 mg/day for 2 wk)	Neuropsychiatric Inventory score, subscores for agitation, aberrant motor, and nighttime behaviors	Significant improvement in Neuropsychiatric Inventory total score, subscores for agitation, aberrant motor, and nighttime behaviors	Minimal	Walther et al. (2006)

TABLE 1
Continued

Disease/Condition	Sample Size, Design, Target Symptoms	Compound (Dose)	Parameters Studied	Results	Adverse Effects	Reference
Primary dystonia						
Primary dystonia	15 patients, DB, R, PL, C, dystonia	Nabilone (0.03 mg/kg)	Motor symptoms	No improvement	Minimal	Fox et al. (2002b)
Tourette's syndrome						
Tourette's syndrome	12 patients, R, DB, PL, C, behavioral disorders	THC (one dose of 5, 7.5, or 10 mg)	Vocal and motor tics, various clinical scales	Significant improvement of vocal and motor tics	Minimal	Müller-Vahl et al. (2002)
Tourette's syndrome	24 patients, R, DB, PL, behavioral disorders	THC (5–10 mg)	Vocal and motor tics, various clinical scales	Significant improvement of vocal and motor tics	Minimal	Müller-Vahl et al. (2003a)
Psychosis/schizophrenia/schizoaffective disorder						
Schizophrenia/ schizoaffective disorder	481 patients, PL, DB, positive and negative symptoms	SR141716; SR 142801 (NK_3 R antagonist); SR46349B (5-$HT_{2A/2C}$ R antagonist)	Various symptom scales	No improvement with CB_1 antagonist, slight improvement with NK_3 R and 5-$HT_{2A/2C}$ R antagonist	Minimal	Meltzer et al. (2004)
Obesity						
Obesity, metabolic syndrome	1507 patients, R, DB, PL, weight	Rimonabant (5, 20 mg, 12 mo)	Weight, WC, BP, lipids, insulin, glucose	Weight and WC reduction, improved lipids, glucose tolerance, metabolic syndrome	Mild (nausea, arthralgia, diarrhea)	Van Gaal et al. (2005)
Obesity, metabolic syndrome	1036 patients, R, DB, PL, weight	Rimonabant (5, 20 mg, 12 m)	Weight, waist c, lipids, glucose, insulin, leptin	Weight and WC reduction, improved cardiovascular risk, reduced metabolic syndrome; decreased BP, increased plasma adiponectin	Mild (nausea, anxiety, diarrhea, insomnia)	Després et al. (2005)
Obesity, metabolic syndrome	3045 patients, R, DB, PL, M, weight, cardiometabolic risk factors	Rimonabant (5, 20 mg, 2 yr)	Weight, WC, lipids, glucose, insulin, leptin	Weight and WC reduction, improved cardiovascular risk, reduced metabolic syndrome; weight regained in 2nd year with placebo	Mild (nausea, anxiety, diarrhea, insomnia)	Pi-Sunyer et al. (2006)

DB, double blind; PL, placebo-controlled; EMG, electromyography; SB, single blind; C, crossover; OL, open-label; max, maximum; R, randomized; M, multicenter; VAS, visual analog scale; P, prospective; PL, placebo; FMF, familial Mediterranean fever; NIB, nitrogen analog of THC; NK_3 R, neurokinin 3 receptor; SR142801, (*R*)-(*N*)-[1-[3-[1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl]propyl]-4-phenylpiperidin-4-yl]-*N*-methylacetamide; SR48692, 2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)carbonylamino]tricycle(3.3.1.1)decan-2-carboxylic acid}; SR463493B, 1(*Z*)-[2-(dimethylamino)ethoxyimino]-1(2-fluorophenyl)-3-(4-hydroxyphenyl)-2(*E*)-propene; 5-$HT_{2A/2C}$ R, serotonin 2A/2C receptor, WC, waist circumference; BP, blood pressure.

IV. Future Directions

The length of this review, necessitated by the steady growth in the number of indications for the potential therapeutic use of cannabinoid-related medications, is a clear sign of the emerging importance of this field. This is further underlined by the quantity of articles in the public database dealing with the biology of cannabinoids, which numbered ~200 to 300/year throughout the 1970s to reach an astonishing 5900 in 2004. The growing interest in the underlying science has been matched by a growth in the number of cannabinoid drugs in pharmaceutical development from two in 1995 to 27 in 2004, with the most actively pursued therapeutic targets being pain, obesity, and multiple sclerosis (Hensen, 2005). As in any rapidly growing area of research, not all the leads will turn out to be useful or even valid. Nevertheless, it is safe to predict that new therapeutic agents that affect the activity of the endocannaboinoid system will emerge and become members of our therapeutic armamentarium. The plant-derived cannabinoid preparation Sativex has already gained regulatory approval in Canada for the treatment of spasticity and pain associated with multiple sclerosis, and the CB_1 receptor antagonist rimonabant has been approved in Europe and is awaiting Food and Drug Administration approval in the United States for the treatment of the metabolic syndrome. Undoubtedly, these will be followed by new and improved compounds aimed at the same or additional targets in the endocannabinoid system. However, it may be only after the widespread therapeutic use of such compounds that some important side effects will emerge. Although this occurrence would be undesirable from a health care perspective, such side effects may shed further light on the biological functions of endocannabinoids in health and disease.

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Smoked cannabis for chronic neuropathic pain: a randomized controlled trial

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Abstract

Background: Chronic neuropathic pain affects 1%–2% of the adult population and is often refractory to standard pharmacologic treatment. Patients with chronic pain have reported using smoked cannabis to relieve pain, improve sleep and improve mood.

Methods: Adults with post-traumatic or postsurgical neuropathic pain were randomly assigned to receive cannabis at four potencies (0%, 2.5%, 6% and 9.4% tetrahydrocannabinol) over four 14-day periods in a crossover trial. Participants inhaled a single 25-mg dose through a pipe three times daily for the first five days in each cycle, followed by a nine-day washout period. Daily average pain intensity was measured using an 11-point numeric rating scale. We recorded effects on mood, sleep and quality of life, as well as adverse events.

Results: We recruited 23 participants (mean age 45.4 [standard deviation 12.3] years, 12 women [52%]), of whom 21 completed the trial. The average daily pain intensity, measured on the 11-point numeric rating scale, was lower on the prespecified primary contrast of 9.4% v. 0% tetrahydrocannabinol (5.4 v. 6.1, respectively; difference = 0.7, 95% confidence interval [CI] 0.02–1.4). Preparations with intermediate potency yielded intermediate but nonsignificant degrees of relief. Participants receiving 9.4% tetrahydrocannabinol reported improved ability to fall asleep (easier, $p = 0.001$; faster, $p < 0.001$; more drowsy, $p = 0.003$) and improved quality of sleep (less wakefulness, $p = 0.01$) relative to 0% tetrahydrocannabinol. We found no differences in mood or quality of life. The most common drug-related adverse events during the period when participants received 9.4% tetrahydrocannabinol were headache, dry eyes, burning sensation in areas of neuropathic pain, dizziness, numbness and cough.

Conclusion: A single inhalation of 25 mg of 9.4% tetrahydrocannabinol herbal cannabis three times daily for five days reduced the intensity of pain, improved sleep and was well tolerated. Further long-term safety and efficacy studies are indicated. (International Standard Randomised Controlled Trial Register no. ISRCTN68314063)

Chronic neuropathic pain has a prevalence of 1%–2%,[1] and treatment options are limited.[2] Pharmacotherapy includes anticonvulsants, antidepressants, opioids and local anesthetics,[3,4] but responses vary and side effects limit compliance.

Cannabis sativa has been used to treat pain since the third millennium BC.[5] An endogenous pain-processing system has been identified, mediated by endogenous cannabinoid ligands acting on specific cannabinoid receptors.[6] These findings, coupled with anecdotal evidence of the analgesic effects of smoked cannabis,[7] support a reconsideration of cannabinoid agents as analgesics.

Oral cannabinoids such as tetrahydrocannabinol, cannabidiol and nabilone have, alone and in combination, shown efficacy in central[8,9] and peripheral[10] neuropathic pain, rheumatoid arthritis[11] and fibromyalgia.[12]

The analgesic effects of smoked cannabis remain controversial, although it is used by 10%–15% of patients with chronic noncancer pain[13] and multiple sclerosis.[14] Clinical trials are needed to evaluate these effects, given that the risks and benefits of inhaled cannabinoids may differ from oral agents. To date, three small clinical trials of the analgesic efficacy of smoked cannabis have been reported.[15–17] All studies were conducted in residential laboratories, and participants smoked multiple doses of the drug at each time point. No study adequately reported data related to adverse events.

We conducted a clinical trial using a standardized single-dose delivery system to explore further the safety and efficacy of smoked cannabis in outpatients with chronic neuropathic pain.

Methods

Participants

The study was approved by the McGill University Health Centre Research Ethics Committee, and all participants gave written informed consent. Participants were recruited at the McGill University Health Centre.

Those eligible were men and women aged 18 years or older with neuropathic pain of at least three months in duration caused by trauma or surgery, with allodynia or hyperalgesia,

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and with an average weekly pain intensity score greater than 4 on a 10-cm visual analogue scale. Participants had a stable analgesic regimen and reported not having used cannabis during the year before the study (Appendix 1, available at www.cmaj.ca/cgi/content/full/cmaj.091414/DC1). Potential participants had to have normal liver function (defined as aspartate aminogransferase less than three times normal), normal renal function (defined as a serum creatinine level < 133 μmol/L), normal hematocrit (> 38%) and a negative result on β human chorionic gonadotropin pregnancy test (if applicable). Women of child-bearing potential consented to use adequate contraception during the study and for three months afterward.

Exclusion criteria were pain due to cancer or nociceptive causes, presence of significant cardiac or pulmonary disease, current substance abuse or dependence (including abuse of or dependence on cannabis), history of psychotic disorder, current suicidal ideation, pregnancy or breastfeeding, participation in another clinical trial within 30 days of enrolment in our trial, and ongoing insurance claims.

Study design

We used a randomized, double-blind, placebo-controlled, four-period crossover design. Each period was 14 days in duration, beginning with five days on the study drug followed by a nine-day washout period. Eligible participants were randomized to a sequence of treatment periods based on a Latin square design.

Cannabis was obtained from Prairie Plant Systems Inc. (Saskatoon, Sask.) and the United States National Institute of Drug Abuse. Prairie Plant Systems Inc. blended cannabis flowers and leaves to prepare three different potencies of active drug (2.5%, 6.0% and 9.4% tetrahydrocannabinol). The US National Institute of Drug Abuse used ethanolic extraction of cannabinoids to prepare the 0% tetrahydrocannabinol product. Intermediate doses (2.5% and 6.0% tetrahydrocannabinol) were used to increase the likelihood of successful blinding. Doses of 25 mg (± 1 mg) were prepared in opaque gelatin capsules by the study pharmacist. A panel of nine independent personnel examined the appearance of the four cannabis preparations and found no association between estimated and true potency (data not shown).

Cannabis doses were delivered as single smoked inhalations using a titanium pipe (RayDiaTor, Mori Designs, Auburn, WA, USA). The first dose of each period was self-administered under observation in a ventilated room. For dose delivery, one capsule of the assigned potency was opened and the cannabis tipped into the bowl of the pipe. Participants were instructed to inhale for five seconds while the cannabis was lit, hold the smoke in their lungs for ten seconds, and then exhale. The beginning of inhalation was recorded as the onset of the exposure. Subsequent doses were self-administered in the same manner three times daily at home for the first five days of each period.

Routine medications were continued throughout the trial. Use of breakthrough analgesia (acetaminophen) was allowed.

Study protocol

The study nurse explained the study to each participant, sought signed informed consent, obtained a medical history and performed a chart review. The study physician conducted a physical examination. Urinary drug screening was performed. Participants were contacted by telephone on three occasions during the first five days of the screening phase to calculate a baseline average pain score. A psychological evaluation was conducted by a clinical psychologist.

On the first day of each period, participants were followed for three hours. Vital signs and ratings of pain, "high," relaxation, stress, happiness and heart rate were recorded, and blood was collected for tetrahydrocannabinol assays. On days one and five of each study period, blood was collected for hematologic and biochemical analyses. At the end of their first visit, participants were given four labelled containers for urine collection and 13 cannabis doses for the five days of treatment.

During the first five days of each period, participants were contacted daily by telephone to administer questionnaires on pain intensity, sleep, medication and adverse effects. Participants collected early morning urine samples daily. They returned on day five to return the urine samples, to undergo urinary and blood tests, and to complete questionnaires on pain quality, mood, quality of life and assessments of potency. At the end of the study, participants completed final adverse event reports and potency assessments. Participants were advised not to drive a vehicle or operate heavy machinery while under the influence of the study drug.

Outcome measures

Outcome measures were selected following published recommendations for clinical trials of chronic pain.[18] Pain intensity was measured using an 11-item numeric rating scale, with "no pain" and "worst pain possible" as anchors. The numeric rating scale was administered once daily for present, worst, least and average pain intensity during the previous 24 hours. As per protocol, the average pain intensity score over the five days on study drug constituted the primary outcome. Acute effects on pain intensity were measured using a 100-mm visual analogue scale. Pain quality was assessed using the McGill Pain Questionnaire.[19] Sleep was assessed using the Leeds Sleep Evaluation Questionnaire.[20] The short-form Profile of Mood States was used to examine mood effects.[21] Quality of life was assessed using the EQ-5D health outcome instrument.[22] The items "high," "relaxed," "stressed," and "happy" were measured using a 100-mm visual analogue scale (0 = not at all, 10 = extremely).[23–25] Potency assessments were conducted by asking participants on the fifth day of each period to guess which potency they had received. At the end of the trial, participants were asked to guess the order in which they received the treatments. Standard assays for plasma tetrahydrocannabinol assays were used (Appendix 1).

Statistical analysis

Our primary hypothesis was that smoked cannabis containing 9.4% tetrahydrocannabinol is superior to 0% tetrahydrocannabinol in reducing average pain intensity. The comparison of within-patient average weekly pain intensity when assigned 9.4% tetrahydrocannabinol cannabis compared with placebo was the contrast of primary interest. A sample size of 32 patients was targeted assuming a within-patient difference

of 10 mm[26] in the primary outcome between active and placebo drug, on a 100 mm scale, with a standard deviation of 20 mm, and with 80% power and 5% significance.

A generalized linear model including drug, period and first-order carryover effects was fitted. If the carryover effect or period effect was not significant, then a reduced model was refitted. Nine-five per cent confidence intervals were generated. Significance tests were performed at a 5% level. An identical procedure to that described above for the primary outcome was performed to assess the secondary outcomes, including the McGill Pain Questionnaire, the Leeds Sleep Evaluation Questionnaire, the Profile of Mood States, and EQ-5D. Statistical procedures for day one assessments and EQ-5D analyses are shown in Appendix 1. Data from all randomized participants were included in all safety and efficacy analyses.

All reported adverse events were classified according to severity, seriousness and relationship to the study drug. An independent data-monitoring committee monitored the safety-related aspects of the trial.

Regulatory considerations

In conducting the study, we followed the Good Clinical Practice guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.[27] The trial was registered with the International Standard for Randomised Controlled Trials Register (ISRCTN683140063).

Results

Participants

We screened 116 potential participants over a 30-month period (August 2003 to January 2006), of whom 93 were ineligible. Twenty-three participants underwent random assignment to treatment, of whom 21 completed all four cycles. Two participants withdrew within the first five days of the study; one (who was receiving placebo at the time) withdrew because of a positive result on urinary screening for cannabinoid and the other (who was receiving 6% tetrahydrocannabinol at the time) because of increased pain (Figure 1). Demographic and baseline pain characteristics of participants are shown in Table 1.

Primary outcome

We found no evidence of significant carryover or period effects for any outcome. The average daily pain intensity was significantly lower on 9.4% tetrahydrocannabinol cannabis (5.4) than on 0% tetrahydrocannabinol (6.1) ($p = 0.023$; difference = 0.7, 95% CI 0.02–1.4). All pairwise differences between groups are shown with 95% CIs in Table 2. The average daily pain scores for each level of tetrahydrocannabinol, along with other secondary outcomes, are shown in Table 2.

Secondary outcomes

There was a trend toward improvement in all outcomes with increasing tetrahydrocannabinol content (Table 3). Participants using 9.4% tetrahydrocannabinol cannabis reported significantly more drowsiness and reported getting to sleep more easily, faster and with fewer periods of wakefulness compared with those using placebo ($p < 0.05$). Anxiety and depression were improved in the 9.4% tetrahydrocannabinol

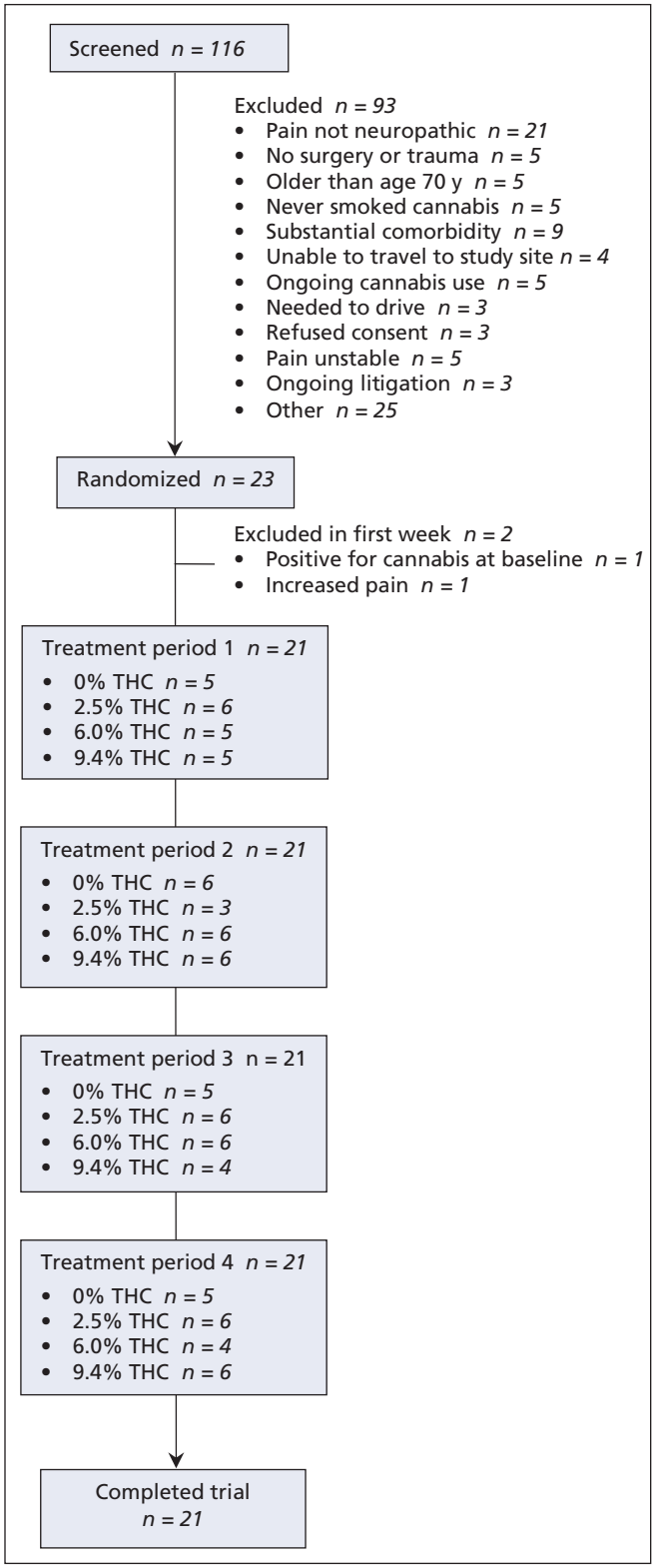

Figure 1: Flow of patients through the randomized controlled trial.

Table 1: Demographic and baseline characteristics of participants

Characteristic	No. (%) of subjects* *n = 23*
Age, yr	
Mean (SD)	45.4 (12.3)
Range	25–77
Sex	
Male	11 (47.8)
Female	12 (52.2)
Education	
Primary or elementary	1 (4.3)
Secondary or high school	8 (34.8)
University or college	14 (60.9)
Employment status	
Full-time or part-time	4 (17.4)
Retired	2 (8.7)
Short-term disability or disabled	14 (60.9)
Other	3 (13.0)
Medications	
Opioids	14 (61)
Antidepressants	12 (52)
Anticonvulsants	10 (43)
NSAIDS	10 (43)
Tobacco use	
Never smoked	8 (34.8)
Current smoker	9 (39.1)
Ex-smoker	6 (26.1)
Ever used alcohol	
Yes	14 (60.9)
No	9 (39.1)
Ever used cannabis	
Yes	18 (81.8)
No	4 (18.2)
Average daily pain at baseline	
Mean (SD)	6.89 (1.37)
Range	4.0–9.2

Note: NSAIDS = nonsteroidal anti-inflammatory drugs, SD = standard deviation.
*Unless otherwise indicated.

group compared with placebo on the EQ-5D subscale ($p < 0.05$). No significant differences were noted on the Profile of Mood States. No difference in the "high," "happy," "relaxed" or "stressed" scores on the visual analogue scale were observed between tetrahydrocannabinol potencies.

A total of 248 mild and six moderate adverse events (fall,[2] increased pain,[1] numbness,[1] drowsiness[1] and pneumonia[1]) were reported during the trial (Table 4). No serious or unexpected adverse events were reported. The total number of adverse events and the number of participants reporting at least one adverse event increased with tetrahydrocannabinol potency. The most frequent drug-related adverse events reported in the group receiving 9.4% tetrahydrocannabinol were headache, dry eyes, burning sensation, dizziness, numbness and cough. Feeling "high" and euphoria were reported once in each of the 2.5%, 6% and 9.4% tetrahydrocannabinol periods. No significant changes in vital signs, heart-rate variability, hematological, biochemistry or renal function blood tests were detected.

On day five of the first cycle, 1 of 5 participants (20%) assigned to placebo correctly identified this assignment, while 9 of the 16 participants (56%) who received placebo during later cycles did so. Of the 5 participants administered 9.4% tetrahydrocannabinol in their first cycle, none correctly identified this assignment, while 10 of 16 patients (63%) did so during later cycles. At the end of the trial, 16 (76%) of the participants were able to correctly identify the 9.4% tetrahydrocannabinol period and 13 (62%) were able to identify the 0% tetrahydrocannabinol period, whereas the 6% tetrahydrocannabinol period was identified by 8 participants (38%) and the 2.5% period by 7 (33%).

Compliance with the study was excellent, and all dispensed capsules were returned. With the exception of one participant who withdrew from the study, there were no positive urine tetrahydrocannabinol tests during the 0% tetrahydrocannabinol period or on any day one before exposure (Appendix 1).

Plasma tetrahydrocannabinol assays revealed dose–response pharmacokinetics (Figure 2) and confirmed that participants did not use cannabis during placebo phases (Appendix 1).

Pharmacy dispensing was satisfactory. No legal issues arose during the study and there were no reports or allegations of diversion of the study drug.

Discussion

We found that 25 mg herbal cannabis with 9.4% tetrahydrocannabinol, administered as a single smoked inhalation three

Table 2: Pairwise comparisons of the effects of four potencies of smoked cannabis on average daily pain

Potency, % of THC	Potency, % of THC, mean difference (95% CI)							
	0		2.5		6.0		9.4	
0	–	–	–	–	–	–	–	–
2.5	–0.13	(–0.83 to 0.56)	–	–	–	–	–	–
6.0	–0.09	(–0.78–0.60)	0.04	(–0.64 to 0.73)	–	–	–	–
9.4	–0.71	(–1.40 to –0.02)	–0.58	(–1.27 to 0.11)	–0.63	(–1.30 to 0.06)	–	–

Note: CI = confidence interval, THC = tetrahydrocannabinol.

Table 3: Effects of smoked cannabis and secondary outcomes, by potency of tetrahydrocannabinol (THC) received

Outcome	Potency of THC, %; outcome measure, mean (SD)*			
	0	2.5	6.0	9.4
Pain intensity				
Average daily pain	6.1 (1.6)	5.9 (1.9)	6.0 (1.8)	5.4 (1.7)†
Highest daily pain	7.1 (1.4)	7.0 (1.6)	7.0 (1.5)	6.5 (1.6)
Lowest daily pain	5.1 (2.1)	5.0 (2.4)	4.8 (2.4)	4.4 (2.2)
McGill Pain Questionnaire				
Sensory	17.2 (10.5)	17.1 (9.9)	14.8 (9.2)	15.6 (8.7)
Affective	3.5 (3.0)	3.8 (3.6)	3.3 (3.4)	3.0 (3.1)
Evaluative	2.2 (1.5)	2.8 (1.3)	2.1 (1.5)	1.7 (1.5)
Miscellaneous	6.2 (4.3)	6.8 (4.4)	5.5 (2.9)	4.5 (3.6)
Total score	29.1 (17.0)	30.4 (18.1)	25.8 (14.5)	24.8 (14.7)
Present pain intensity	2.8 (1.2)	3.0 (1.0)	2.8 (1.0)	2.5 (1.1)
Leeds Sleep Evaluation Questionnaire‡				
Getting to sleep				
Harder — easier than usual	5.4 (1.5)	5.5 (1.6)	6.1 (1.5)	6.8 (1.8)†
Slower — faster than usual	5.3 (1.3)	5.6 (1.4)	6.2 (1.7)	6.9 (1.7)†
Less — more drowsy than usual	5.3 (1.1)	5.9 (1.4)	5.7 (1.3)	6.6 (1.5)†
Quality of sleep				
More restless — more restful	5.5 (1.6)	5.4 (1.7)	5.9 (2.0)	6.5 (2.1)
More — less period wakefulness than usual	5.3 (1.5)	5.0 (1.5)	5.5 (1.7)	6.3 (1.8)†
Awakening this morning				
More difficult — easier	4.6 (1.2)	4.4 (0.8)	4.7 (1.4)	4.8 (1.0)
Took longer — shorter	4.4 (0.8)	4.4 (0.9)	1.7 (1.1)	5.0 (1.0)
Feeling on waking-up				
Tired — alert	4.3 (1.9)	4.0 (1.5)	5.2 (1.9)	4.9 (1.9)
Feeling now				
Tired — alert	4.1 (1.5)	1.3 (1.7)	4.9 (2.0)	4.0 (1.7)
Sense of balance				
More — less clumsy than usual	4.9 (0.4)	4.8 (0.4)	4.9 (0.4)	5.0 (1.2)
EQ-5D health outcomes§				
Mobility, no. (%)	10 (48)	11 (52)	11 (52)	11 (55)
Self-care, no. (%)	14 (67)	12 (57)	15 (71)	14 (70)
Usual activities, no. (%)	3 (14)	3 (14)	4 (19)	5 (25)
Pain or discomfort, no. (%)	11 (52)	10 (48)	14 (67)	14 (75)
Anxiety or depression, no. (%)	4 (19)	5 (23)	7 (33)	9 (45)†
State of health, no. (%)	3 (14)	2 (9)	4 (19)	7 (35)
State of health (VAS)	54.1 (19.5)	48.6 (18.9)	52.9 (22.0)	56.3 (20.4)
Profile of Mood States (POMS)¶				
Depression	10.6 (6.5)	10.4 (6.7)	9.3 (6.6)	9.4 (5.7)
Vigour	7.3 (4.3)	7.3 (5.4)	6.2 (4.6)	8.0 (4.6)
Anger	9.2 (7.0)	7.7 (6.3)	7.9 (7.6)	6.5 (6.0)
Tension	8.5 (5.1)	9.3 (4.6)	9.0 (5.6)	7.2 (5.2)
Confusion	6.3 (3.7)	6.7 (4.0)	6.0 (4.3)	5.7 (4.1)
Fatigue	11.9 (4.1)	11.1 (5.0)	11.1 (4.8)	10.5 (5.0)
Total mood disturbance	39.1 (22.7)	38.0 (24.5)	36.9 (25.9)	31.2 (22.4)

Note: EQ-5D = health outcome instrument,[22] SD = standard deviation, VAS = visual analog scale.
*Unless indicated otherwise.
†$p < 0.05$ for the comparison with 0% THC.
‡Higher scores indicate improved sleep parameters.
§Data are presented as a proportion of subjects reporting the most favourable responses; thus, a higher proportion suggests a better health outcome.
¶With the exception of vigour, lower scores represent better mood.

Table 4: Adverse events reported during the study, by potency of tetrahydrocannabinol (THC) (part 1 of 2)

Adverse event	% of THC 0 *n* = 21	2.5 *n* = 22	6.0 *n* = 21	9.4 *n* = 22
Nervous system disorders				
Asthenia	1	3	0	2
Decreased motor skill	0	0	0	1
Dizziness	2	3	4	4
Drowsiness	1	2	2	0
Headache	3	3	7	4
Heavy-headed	0	0	0	1
Insomnia	1	1	1	0
Lethargic	0	0	1	0
Lightheaded	1	1	0	1
Migraine	0	1	0	0
Nightmare	1	0	0	0
Not sleeping well	1	0	0	0
Numbness	1	2	1	2
Sleepiness	0	0	1	2
Spasm	1	0	0	0
Tiredness	1	1	1	0
Unbalanced	0	1	0	1
Total	**14**	**18**	**18**	**18**
General disorders and conditions specific to site of administration				
Bad taste in oral cavity	1	1	0	0
Burning sensation	3	2	3	3
Cheeks flushed	0	0	1	0
Chills	1	2	1	0
Diaphoresis	1	0	0	0
Fall	2	1	0	0
Fatigue	2	3	3	2
Heaviness	0	2	0	1
Hematoma	0	0	0	1
Irritation of oral cavities	0	0	0	1
Itchiness	0	0	0	1
Itchiness in face	0	0	0	1
Itchiness of nose	0	0	2	1
Pain	2	2	3	2
Tingling nose	0	0	1	1
Total	**12**	**13**	**14**	**13**
Psychiatric disorders				
Anxiety	0	0	1	0
Craving for sweets	0	0	0	1
Disinterest in surroundings	0	0	0	1
Dysphoria	0	0	0	2
Euphoria	0	1	0	1
Feel high	0	0	1	0
Fidgety fingers	0	0	0	1
Foggy mental state	0	0	1	1
Lack of concentration	1	2	2	2
Less alert	0	0	0	1
Lost in time	0	1	0	0
Paranoia	0	0	0	1
Racing thoughts	0	0	0	1
Stressful	0	1	0	0
Total	**1**	**5**	**5**	**12**

Adverse event	% of THC 0 *n* = 21	2.5 *n* = 22	6.0 *n* = 21	9.4 *n* = 22
Psychiatric disorders (continued)				
Feel high	0	0	1	0
Fidgety fingers	0	0	0	1
Foggy mental state	0	0	1	1
Lack of concentration	1	2	2	2
Less alert	0	0	0	1
Lost in time	0	1	0	0
Paranoia	0	0	0	1
Racing thoughts	0	0	0	1
Stressful	0	1	0	0
Total	**1**	**5**	**5**	**12**
Respiratory, thoracic and mediastinal disorders				
Cough	1	1	3	3
Pneumonia	1	0	0	0
Short of breath	0	0	1	1
Throat irritation	3	4	3	3
Total	**5**	**5**	**7**	**7**
Gastrointestinal disorders				
Decreased appetite	1	0	1	0
Dry mouth	0	0	0	1
Gastric acid	0	0	1	0
Increased appetite	0	1	1	2
Loss of appetite	0	1	0	0
Nausea	1	2	2	1
Thirst	0	0	1	0
Vomiting	0	1	0	0
Total	**2**	**5**	**6**	**4**
Ear and labyrinth disorders				
Ear buzzing	0	0	1	0
Total	**1**	**0**	**1**	**0**
Eye disorders				
Blurry vision	1	0	0	0
Dry eyes	0	0	0	1
Eyes red	0	0	1	0
Itchiness of eyes	0	1	2	1
Total	**1**	**1**	**3**	**2**
Musculoskeletal and connective tissue disorders				
Achy bones	0	1	0	0
Bruise on left back shoulder	1	0	0	0
Edema	1	0	0	1
Heaviness in leg	0	0	1	0
Injury to right knee	0	0	0	1
Muscles of jaw contracted	0	0	1	0
Musculoskeletal pain	1	0	0	0
Weakness of right leg	1	0	0	0
Total	**4**	**1**	**2**	**2**

continued

Table 4: Adverse events reported during the study, by potency of tetrahydrocannabinol (THC) (part 2 of 2)

Adverse event	% of THC 0 *n = 21*	2.5 *n = 22*	6.0 *n = 21*	9.4 *n = 22*
Infections and infestations				
Fever	0	1	0	0
Total	**0**	**1**	**0**	**0**
Renal and urinary disorders				
Difficulty voiding	0	1	0	0
Total	**0**	**1**	**0**	**0**
Disorders of skin and subcutaneous tissue				
Rash	0	0	0	1
Total	**0**	**0**	**0**	**1**
Surgical and medical procedures				
Minor surgery	1	0	0	0
Total	**1**	**0**	**0**	**0**
Total adverse events	**46**	**61**	**65**	**82**

Note: THC = tetrahydrocannabinol.

times daily for five days, significantly reduced average pain intensity compared with a 0% tetrahydrocannabinol cannabis placebo in adult participants with chronic post-traumatic or postsurgical neuropathic pain. We found significant improvements in measures of sleep quality and anxiety. We have shown the feasibility of a single-dose delivery method for smoked cannabis, and that blinding participants to treatment allocation is possible using this method.

The mean reduction in pain (0.7) from 6.1 to 5.4 on a 10-cm scale that we detected in this study is modest when compared with that from other drugs for chronic neuropathic pain, such as gabapentin (1.2) and pregabalin (1.3).[28,29] However, our study involved participants with refractory pain for which conventional therapies had failed, and this characteristic may have limited the potential for findings of a larger pain reduction.

The effects of cannabinoids on sleep are recognized.[7,9] The consistent trend toward improvement in all other outcomes for 9.4% tetrahydrocannabinol compared with placebo in our trial suggests that the reported effects on pain, mood and sleep may have been part of an overall improvement in many aspects of patients' conditions.

Limitations and strengths

There were several limitations to this trial. The number of participants recruited was smaller than planned, owing to delays in obtaining licences, approvals and the study drug, and to restrictive criteria for eligibility. Most of our participants had prior experience with cannabis, which had been an early ethics requirement; none was using cannabis at the time of enrolment and they were not "experienced" users, so that the lessons learned would be applicable to naive users of medical cannabis. The use of small, fixed doses with a short trial duration may have reduced the effect size. We used a low dose to minimize exposure to smoke and to reduce psychoactive effects. Previous work has shown that a single dose of 0.4 mg/kg can be inhaled in a single lungful from a pipe,[24,30] which for a 70-kg person approximates to 25 mg per dose. The frequency of dosing was based on a duration of action of inhaled tetrahydrocannabinol of two to three hours[31] and was administered three times daily. We used a fixed dosing schedule because the study was too short to allow dose titration and we wanted the tetrahydrocannabinol potency to be the only difference between cycles. Finally, the highest tetrahydrocannabinol-content cannabis (9.4%) legally available at the time of the study was used. Additional studies with higher potencies and flexible dosing strategies are needed to explore dose–response effects.

With respect to our analysis, we are aware of issues surrounding the use of early tests for carryover effects. However, examination of pain scores during the washout period showed that the washout was adequate (data not shown), and therefore we believe our approach was appropriate.

Our trial had several important strengths, including a credible placebo, good compliance and good safety reporting. Finding a suitable placebo for smoked cannabis is not a trivial issue. During protocol reviews, it was stated that participants smoking cannabis would immediately know, based on the acute psychoactive effects, whether they had received active drug; however, our results do not support this view. Instead, our data suggest that short-term placebo-controlled trials of smoked cannabis are feasible.

The safety of smoked cannabis is a concern for patients and

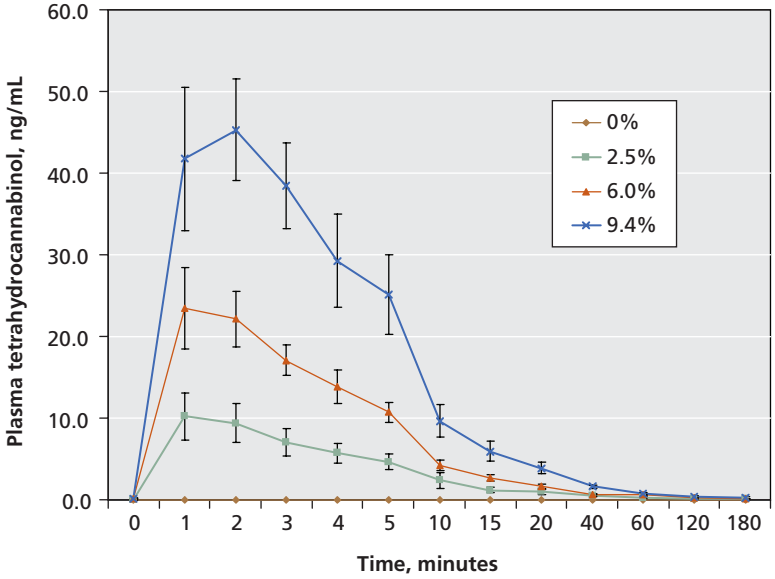

Figure 2: Levels of tetrahydrocannabinol (THC) in plasma after inhalation of a single dose. Data are presented as means and standard deviations.

physicians, and we made a concerted effort to collect data on adverse events and describe short-term physiologic effects. The frequency of adverse events increased with tetrahydrocannabinol potency. Psychoactive effects did not result in participants withdrawing from the study. Euphoria or "high" was reported on only three occasions throughout the trial. There was no evidence of euphoria during the three hours following the first dose of each cycle regardless of tetrahydrocannabinol potency, possibly because plasma levels (mean 45 ng/mL) did not reach levels found with recreational users (> 100 ng/mL).[31]

Conclusion

Our results support the claim that smoked cannabis reduces pain, improves mood and helps sleep. We believe that our trial provides a methodological approach that may be considered for further research. Clinical studies using inhaled delivery systems, such as vaporizers,[32,33] are needed.

This article has been peer reviewed.

Competing interests: None declared.

Contributors: Mark Ware conceived and designed the study, and drafted the manuscript. Stan Shapiro, Jean-Paul Collet, Thierry Ducruet and Gary Bennett were involved in the conception and design of the study. Thierry Ducruet, Ann Robinson, Ann Gamsa and Thao Huynh were involved in the acquisition, analysis and interpretation of data. Tongtong Wang performed analysis of the data with support from Thierry Ducruet and Stan Shapiro. All of the authors were involved in the critical revision of the manuscript, and all of them approved the final draft submitted for publication.

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Cannabinoid–Opioid Interaction in Chronic Pain

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Cannabinoids and opioids share several pharmacologic properties and may act synergistically. The potential pharmacokinetics and the safety of the combination in humans are unknown. We therefore undertook a study to answer these questions. Twenty-one individuals with chronic pain, on a regimen of twice-daily doses of sustained-release morphine or oxycodone were enrolled in the study and admitted for a 5-day inpatient stay.Participants were asked to inhale vaporized cannabis in the evening of day 1, three times a day on days 2–4, and in the morning of day 5. Blood sampling was performed at 12-h intervals on days 1 and 5. The extent of chronic pain was also assessed daily. Pharmacokinetic investigations revealed no significant change in the area under the plasma concentration–time curves for either morphine or oxycodone after exposure to cannabis. Pain was significantly decreased (average 27%, 95% confidence interval (CI) 9, 46) after the addition of vaporized cannabis. We therefore concluded that vaporized cannabis augments the analgesic effects of opioids without significantly altering plasma opioid levels. The combination may allow for opioid treatment at lower doses with fewer side effects.

Selecting an appropriate treatment for chronic pain remains problematic. Although opioids are effective analgesics, dose-limiting side effects such as sedation, nausea and vomiting, and fear of dependence often limit their use at higher—and possibly more effective—doses. Of particular interest is the potential for enhanced analgesic effect with the use of cannabinoids and opioids in combination. Such a combination would allow for opioid analgesic effects to be achieved at lower dosages than are necessary when the opioids are used alone.[1–4] As increasing numbers of patients turn to medicinal cannabis to augment the effects of opioid analgesics, the data on the potential pharmacokinetic interactions and clinical safety of the combination need to be evaluated.

Cannabinoids and opioids share several pharmacologic properties, including antinociception; a tendency to induce hypothermia, sedation, and hypotension; and inhibition of intestinal motility and locomotor activity.[1,5,6] Initially, investigators postulated that cannabinoids and opioids act on the same pathways to produce their pharmacological actions.[7,8] Subsequent preclinical research conducted over the past decade has clarified the nature of the interaction; these data suggest the existence of independent but related mechanisms of antinociception for cannabinoids and opioids.[5]

Synergy in analgesic effects between opioids and cannabinoids has been demonstrated in animal models. The antinociceptive effects of morphine are mediated predominantly by mu opioid receptors but may be enhanced by delta-9-tetrahydrocannabinol (THC) activation of kappa and delta opiate receptors.[8] It has further been suggested that the cannabinoid–opioid interaction may occur at the level of their signal transduction mechanisms.[9,10] Receptors for both classes of drugs are coupled to similar intracellular signaling mechanisms that lead to a decrease in cyclic adenosine monophosphate production via G protein activation.[10–12] There is also some evidence that cannabinoids increase the synthesis and/or release of endogenous opioids.[2,3,12,13]

In addition to these potential pharmacodynamic interactions, there is the potential for pharmacokinetic interaction between cannabinoids and other drugs. Cannabinoids have been shown to affect the kinetics of other drugs in several ways. They inhibit the CYP450-mediated metabolism of some drugs, slow the absorption of others, and may also enhance penetration of some drugs into the brain.[14–16] Our prior study of oral delta-9-THC and smoked cannabis in patients with HIV on protease inhibitor therapies showed that oral THC had no effect on the pharmacokinetics of the antiviral agents.[17] However, smoked cannabis decreased the 8-h area under the plasma concentration–time curve (AUC) of both nelfinavir (−17.4%, $P = 0.46$) and indinavir (−14.5%, $P = 0.07$). In a study involving 24 patients with cancer, cannabis administered as a medicinal tea did not alter the pharmacokinetics of the chemotherapy agents irinotecan and docetaxel.[18]

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Inhalation of vaporized cannabis delivers levels of THC and other cannabinoids similar to those from smoked marijuana but without exposure to combustion products.[19] Here we describe the disposition kinetics of sustained-release morphine and oxycodone, as well as pain ratings and other subjective responses, before and after 4 days of treatment with vaporized cannabis.

RESULTS

Study participants

A total of 315 potential participants were assessed for eligibility between January 2007 and February 2009; most of them were deemed ineligible because they either did not have pain, were not taking the appropriate opioids, or were receiving opioids three times a day. A total of 24 participants were enrolled, 13 of whom were on morphine treatment and 11 on oxycodone. Of those on morphine, 3 participants did not complete the study, leaving 21 evaluable participants (10 on morphine, and 11 on oxycodone) (see **Table 1**). Most of the participants (11 men and 10 women) were white. The average age was 42.9 (range = 33–55) years in the morphine cohort and 47.1 (range = 28–61) years in the oxycodone cohort. The mean morphine dose was 62 mg twice a day (range = 10–200 mg) and the mean oxycodone dose was 53 mg twice a day (range = 10–120 mg). The origin of the participants' pain was musculoskeletal (not otherwise specified) (seven); posttraumatic (four); arthritic (two); peripheral neuropathy (two); cancer, fibromyalgia, migraine, multiple sclerosis, sickle cell disease, and thoracic outlet syndrome (one each).

Pain

Pain ratings on day 1 (before exposure to vaporized cannabis) and on day 5 (after exposure to vaporized cannabis) are shown in **Table 2**. Participants on oxycodone had higher mean pain scores at baseline (mean = 43.8; 95% confidence interval (CI) = 38.6, 49.1) compared with those on morphine (mean = 34.8; 95% CI = 29.4, 40.1). Participants in both groups reported statistically significant reductions in pain ratings on day 5 as compared with day 1. The mean percentage change in pain was statistically significant overall as well as for the patients on morphine, but not for those on oxycodone.

Table 1 Participant characteristics

	Morphine group	Oxycodone group
n	10	11
Women	4	6
Caucasian	8	9
Mean age (range)	42.9 (33–55)	47.1 (28–61)
Mean opioid dose (mg) (range)	62 Twice daily (10–200)	53 Twice daily (10–120)
Mean pain score day 1 (95% CI)	34.8 (29.4, 40.1)	43.8 (38.6, 49.1)

CI, confidence interval.

Opioid disposition kinetics

Mean plasma concentration–time curves for morphine and oxycodone with and without cannabis treatment are shown in **Figure 1**. There was no statistically significant change in the AUC_{12} for either of these opiates (see **Table 3**). There was a statistically significant decrease in maximum concentration (C_{max}) of morphine sulfate during cannabis exposure. The time to C_{max} of morphine tended to be delayed during cannabis treatment, although this effect was not statistically significant. Cannabis had no significant effect on oxycodone kinetics. During cannabis treatment, there were no significant changes in the AUCs of the metabolites of either morphine or oxycodone or in the ratios of individual metabolites to the parent drug.

Plasma THC levels

Mean plasma THC levels were 1.8 ng/ml (SD = 1.5) at baseline, 126.1 ng/ml (SD = 86.2) at 3 min, 33.7 ng/ml (SD = 28.9) at 10 min, 10.9 ng/ml (SD = 9.3) at 30 min, and 6.4 ng/ml (SD = 5.6) at 60 min. The peak THC concentration occurred at 3 min in all the participants. THC plasma levels did not vary significantly by opioid group.

Monitoring of effects

Cannabis inhalation produced a subjective "high" that was not present with the use of opioids alone (see **Figure 2**). In addition, the participants in the morphine cohort felt significantly more stimulated and less hungry on day 5 than on day 1 (see **Table 4**), whereas those in the oxycodone group were less anxious on day 5 as compared with day 1. Other than these, there were no significant changes in the subjective effects measured. No clinically significant adverse events were reported. Pulse oximetry monitoring did not reveal any episodes of lowered oxygen saturation after cannabinoids were added to the participants' stable opioid regimens.

DISCUSSION

Our study findings support preclinical observations that cannabis augments the analgesic effects of opioids. We studied individuals with chronic pain who were taking stable doses of sustained-

Table 2 Pain by study day

		Day 1	Day 5	Difference	Percentage change
	n	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)
Overall	21	39.6 (35.8, 43.3)	29.1 (25.4, 32.8)	−10.7 (−14.4, −7.3)	−27.2 (−45.5, −8.9)
Morphine	11	34.8 (29.4, 40.1)	24.1 (18.8, 29.4)	−11.2 (−16.5, −6.0)	−33.7 (−63.8, −3.5)
Oxycodone	10	43.8 (38.6, 49.1)	33.6 (28.5, 38.6)	−10.3 (−14.8, −5.8)	−21.3 (−47.0, 5.3)

CI, confidence interval.

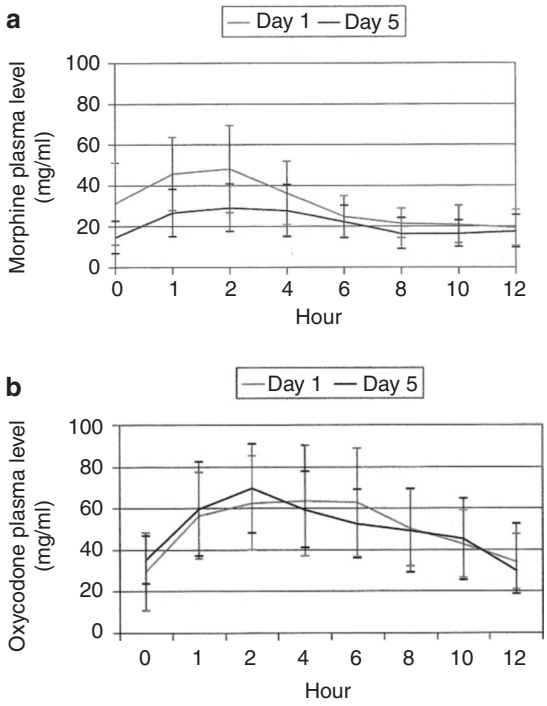

Figure 1 Plasma concentration–time curves for sustained-release (**a**) morphine and (**b**) oxycodone before and after exposure to inhaled cannabis.

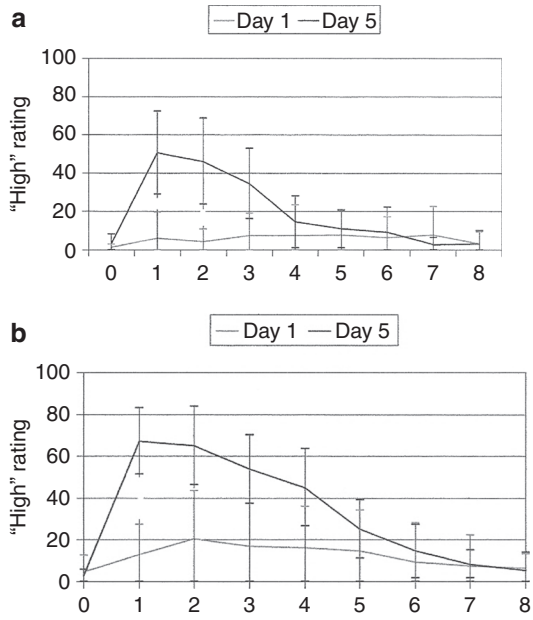

Figure 2 Subjective highs experienced when cannabis was combined with (**a**) morphine and (**b**) oxycodone on day 5.

release morphine or oxycodone. The participants experienced less pain after 5 days of inhaling vaporized cannabis; when the morphine and oxycodone groups were combined, this reduction in pain was significant. This is the first human study to demonstrate that inhaled cannabis safely augments the analgesic effects of opioids. Several other studies have examined the analgesic interaction between oral THC and opioids. Two of those studies involved healthy volunteers exposed to experimental pain conditions.[14,20] THC had little effect in either of the studies, whereas the combination of THC and morphine had synergistic effects on affective responses to pain in one study and on response to electrical stimulation in the other. A placebo-controlled trial in patients taking opioids for chronic pain found that oral dronabinol (delta-9-THC) decreased pain significantly.[15]

The mechanism by which cannabis augments the analgesic effects of opioids could be pharmacokinetic and/or pharmacodynamic. Cannabinoids have been shown to inhibit the metabolism of certain other drugs, both *in vitro* and *in vivo*.[16,21,22] THC has been shown to slow gastrointestinal motility, resulting in the slowing of absorption of orally administered drugs such as pentobarbital and ethanol. THC has also been shown to slow the intranasal absorption of cocaine.[23–25] In animals, cannabinoids have been shown to enhance the uptake of drugs, including cocaine and phencyclidine, into the brain; however, the mechanisms involved are not fully understood.[26]

In the present study, we examined the effects of vaporized cannabis administered three times a day on the steady-state pharmacokinetics of sustained-release morphine and oxycodone administered at 12-h intervals. In the case of morphine, we found that cannabis treatment was associated with a significant decrease in the maximal concentration. On average, the time to maximal morphine concentration was longer during cannabis administration, although this effect was not significant. There were no significant effects of cannabis treatment on the AUCs of morphine's metabolites or on the ratios of metabolites to parent morphine, indicating that cannabis had no effects on metabolic pathways. Vaporized cannabis had no significant effect on oxycodone kinetics or metabolite levels. The finding of a lower maximal concentration of morphine without any accompanying changes in metabolite levels during cannabis treatment is probably due to delayed absorption of morphine, presumably because of slowed gastrointestinal motility. Why such an effect was not seen for oxycodone is not clear. From the pharmacokinetic findings, it is clear that the observed augmentation of analgesia by cannabis cannot be explained on the basis of inhibition of morphine or oxycodone metabolism leading to higher plasma levels of these drugs.

Our findings suggest that cannabis augments opioid analgesia through a pharmacodynamic mechanism. However, prior research in rodents has shown that THC and cannabidiol enhance the penetration of certain other drugs, including cocaine and phencyclidine, into the brain.[26] If cannabinoids also enhance opioid penetration into the brain in humans, this might constitute a pharmacokinetic mechanism for enhancing the analgesic effects of opioids.

The participants reported a subjective high after inhaling cannabis, with little or no high after taking the oral opioids alone. Although we do not have data on the high in these participants in the absence of opioids (that is, with cannabis alone), the magnitude and time course of the high in the participants in the morphine group were similar to our observations in a previous study of inhaled cannabis in healthy subjects.[19] The high in the oxycodone group after cannabis treatment appeared to be more sustained than that in the morphine group, and also as compared with that of our previously studied healthy subjects.

Table 3 Morphine, oxycodone, and their metabolites: mean AUC and CV by study day

	Day 1			Day 5			Day 5/day 1			
	n	Geometric mean	CV	*n*	Geometric mean	CV	Ratio	95% CI	*P* value	
									Par	N-par
Morphine and its metabolites										
Morphine										
T_{max}[a]	10	3.1		10	4.74		1.64	−1.01, 4.30	0.19	0.2
C_{max}	10	43.68	15.95	10	29.66	15.74	0.9	0.85, 0.95	**0.003**	**0.002**
AUC	10	42.01	18.7	10	32.23	15.23	0.95	0.84, 1.05	0.17	0.23
M3g										
C_{max}	10	1,123.94	6.89	10	887.14	4.56	0.97	0.93, 1.00	0.06	0.08
AUC	10	821.39	9.54	10	756.73	7.41	1	0.92, 1.07	0.74	1
M6g										
C_{max}	10	188.67	16.28	10	153.22	6.53	0.97	0.92, 1.01	0.11	0.16
AUC	10	128.25	10.41	10	130.45	10.94	1.02	0.90, 1.15	0.95	0.85
M3g/morphine	10	6.32	17.66	10	6.92	6.92	1.06	0.98, 1.15	0.23	0.19
M6g/morphine	10	3.79	22.69	10	4.13	4.13	1.09	0.98, 1.21	0.25	0.08
Oxycodone and its metabolites										
Oxycodone										
T_{max}[a]	11	3.63		11	2.52		−1.11	−3.66, 1.43	0.35	0.9
C_{max}	11	64.91	12.87	11	62.74	16.67	0.99	0.89, 1.10	0.84	1
AUC	11	76.86	13.38	11	58.67	19.18	0.94	0.84, 1.04	0.18	0.32
Noroxycodone										
C_{max}	11	52.72	14.69	11	65.17	11.78	1.07	0.96, 1.17	0.22	0.46
AUC	11	38.67	15.1	11	36.97	17.11	1.01	0.85, 1.16	0.86	0.7
Oxymorphone										
C_{max}	11	1.42	203.31	11	1.39	175.91	0.15	−1.67, 1.96	0.9	0.82
AUC	10	1.32	334.96	10	1.25	302.37	0.63	0.00, 1.26	0.78	0.77
Noroxycodone/oxycodone	11	2.34	18.33	11	2.49	21.91	1.09	0.93, 1.25	0.31	0.37
Oxymorphone/oxycodone	10	1.07	328.32	10	1.05	354.88	0.7	−0.01, 1.41	0.63	0.63

Statistically significant values are in bold face. AUC, area under the plasma concentration–time curve; CI, confidence interval; C_{max}, maximum concentration; CV, coefficient of variation; M3g, morphine-3-glucuronide; M6g, morphine-6-glucuronide; N-par, nonparametric; Par, parametric; T_{max}, time to maximum concentration.

[a]T_{max} values are expressed as arithmetic means on each study day with standard deviation as the measure of variance. Comparisons of T_{max} values on day 1 and day 5 are expressed as the paired difference in these values (day 5 – day 1).

Our study has some limitations. The number of participants was relatively small, although we were powered to detect a 25% change in the 12-hour AUC (AUC_{12}). With respect to pain assessment, our study was not placebo-controlled, and therefore we cannot rule out the possibility that cannabis-enhanced analgesia was a placebo effect or a time effect of changes in activity levels associated with confinement in the inpatient research ward setting throughout the duration of the study. The intervention we used was vaporized cannabis, which delivers levels of THC and other cannabinoids similar to those of smoked cannabis without exposing the user to the combustion products of cannabis cigarettes, which could affect the metabolism and pulmonary uptake of other drugs. Oral cannabis is commonly used to deliver medicinal THC and results in high first-pass levels of cannabinoids in the liver, which could have effects on opioid metabolism different from those caused by vaporized cannabis. Therefore, further research is needed to determine how different cannabis delivery systems affect the metabolism of opioids and other drugs.

In conclusion, we found that vaporized cannabis augments analgesia in individuals with chronic pain on a treatment regimen of stable doses of sustained-release morphine or oxycodone, and that the mechanism of augmentation is not explained by elevation of plasma opioid concentrations or inhibition of opioid metabolism. Cannabis appears to slow morphine absorption such that maximal concentrations for a dosing interval are lower. The effect of inhaled cannabis in enhancing opiate analgesia is most likely achieved through a pharmacodynamic mechanism. These results suggest that further controlled studies of the synergistic interaction between cannabinoids and opioids are warranted.

Table 4 Subjective effects: morphine vs. morphine/cannabis and oxycodone vs. oxycodone/cannabis

	Day 1			Day 5			Day 5 – day 1		
	n	Mean	SD	*n*	Mean	SD	Difference	95% CI	*P* value
Morphine vs. morphine/cannabis									
Like effect									
C_{max}	9	54.56	24.38	10	63.5	29	6.89	−8.49, 22.26	0.33
AUC	10	2.99	2.99	10	2.01	1.2	−0.98	−3.00, 1.04	0.3
High									
C_{max}	10	13.6	24.57	10	54.7	30.76	41.1	20.85, 61.35	**0.001**
AUC	10	0.74	1.44	10	1.96	1.25	1.22	0.24, 2.20	**0.02**
Stimulated									
C_{max}	10	11.7	23.24	10	37.6	31.91	25.9	9.03, 42.77	**0.007**
AUC	10	0.55	1.08	10	1.5	1.6	0.96	−0.10, 2.01	0.07
Anxious									
C_{max}	10	31.8	27.84	10	27.4	29.33	−4.4	−25.12, 16.32	0.64
AUC	10	1.73	1.84	10	1.29	2.01	−0.44	−2.02, 1.14	0.54
Sedated									
C_{max}	10	36.9	32.42	10	36.5	24.67	−0.4	−21.64, 20.84	0.97
AUC	10	2.75	2.89	10	1.74	1.47	−1.01	−3.03, 1.00	0.29
Hungry									
C_{max}	10	64.8	34.57	10	42	29.44	−22.8	−44.71, −0.89	**0.04**
AUC	10	2.89	2.3	10	1.34	1.28	−1.55	−3.09, −0.02	**0.05**
Dry mouth									
C_{max}	10	32	22.97		25.8	30.75	−6.2	−31.82, 19.42	0.6
AUC	10	2.29	2.34	10	1.28	2.13	−1.01	−3.16, 1.15	0.32
Oxycodone vs. oxycodone/cannabis									
Like effect									
C_{max}	11	62.91	30.03	11	78.27	17.84	15.36	−3.14, 33.86	0.09
AUC	11	2.92	1.74	11	3.21	1.49	0.29	−0.69, 1.28	0.52
High									
C_{max}	11	23.73	29.35	11	72.73	23.22	49	27.82, 70.18	**0.001**
AUC	11	0.96	0.91	11	3.47	1.58	2.5	1.65, 3.36	**0.001**
Stimulated									
C_{max}	11	32.64	32.09	11	30	28.42	−2.63	−23.05, 17.77	0.78
AUC	11	1.21	1.12	11	1.76	2.27	0.55	−0.76, 1.87	0.37
Anxious									
C_{max}	11	49.73	34.04	11	33.39	33.39	−16.45	32.02, 0.89	**0.04**
AUC	11	2.22	1.87	11	1.88	1.88	−0.55	−1.55, 0.46	0.26
Sedated									
C_{max}	11	37.18	32.46	11	30.74	30.74	14.73	−10.06, 39.51	0.22
AUC	11	1.67	1.51	11	1.38	1.38	0.57	−0.96, 2.10	0.42
Hungry									
C_{max}	11	61.18	24.12	11	28.56	28.56	4.1		0.92
AUC	11	3.27	2.33	11	2.15	2.15	−0.5	−2.46, 1.45	0.58
Dry mouth									
C_{max}	11	22.18	19.6	11	33.65	33.65	23.45	−7.38, 54.29	0.12
AUC	11	1	1.07	11	1.32	1.32	0.6	−0.77, 7.97	0.35

Statistically significant values are in bold face. AUC, area under the plasma concentration–time curve; CI, confidence interval; C_{max}, maximum concentration.

METHODS

Study participants. The participants were adults >18 years of age who were experiencing chronic pain and receiving ongoing analgesic therapy with sustained-release morphine sulfate (MS Contin) or oxycodone hydrochloride (OxyContin) every 12 h. The participants were required to have been on a stable medication regimen for at least 2 weeks prior to the commencement of the study. Hepatic transaminase levels were required to be within 5 times the upper limit of normal and serum creatinine to be <2.0 mg/dl (177 μmol/l). A negative pregnancy test was required for female participants. Exclusion criteria included severe coronary artery disease, uncontrolled hypertension, cardiac ventricular conduction abnormalities, orthostatic mean blood pressure drop of >24 mm Hg, severe chronic obstructive pulmonary disease, history of renal or hepatic failure, active substance abuse, neurologic dysfunction or psychiatric disorder severe enough to interfere with assessment of pain, current use of smoked tobacco products or a confirmed cotinine level, and, in women, pregnancy, breastfeeding, or not using adequate birth control.

All the participants were required to have prior experience of smoking cannabis (six or more times in their lifetime) so that they would know how to inhale and what neuropsychologic effects to expect. Current users were asked to discontinue cannabis use for 30 days prior to commencement of the study, and such abstention was confirmed by a negative urine THC assay prior to study enrollment.

The study was approved by the institutional review board at the University of California, San Francisco; the Research Advisory Panel of California; the Drug Enforcement Administration; the US Food and Drug Administration, and the National Institute on Drug Abuse. Written informed consent was obtained from all the participants. The ClinicalTrials.gov registration number was NCT00308555.

Study medication. The National Institute on Drug Abuse provided cannabis in the form of cigarettes weighing 0.9 g on average and containing 3.56% delta-9-THC. The cigarettes were kept in a locked freezer with an alarm device attached until they were dispensed to a locked freezer in the San Francisco General Hospital Clinical Research Center where the inpatient study was conducted. The frozen cigarettes were thawed and rehydrated overnight in a humidifier. The cannabis was removed from the prerolled cigarettes and administered in a Volcano vaporizer (Model #0100 CS; Tuttlingen, Germany), heated to 190°C.[27] The study participants were housed in a room with a fan ventilating to the outside. To maximize standardization of the vaporized doses, the subjects followed a uniform puffing procedure: the cannabis was inhaled for 5 s and then held for 10 s, with a 45-s pause before a repeat inhalation.[28] The participants were encouraged to inhale the entire vaporized dose of 0.9 g of 3.56% delta-9-THC or as much as they could tolerate.

In a previous study we had demonstrated that this vaporization procedure results in plasma THC levels similar to those induced by smoked marijuana but without significant exposure to carbon monoxide and other combustion products.[19]

Opioid disposition kinetics. Opioid pharmacokinetics were determined on days 1 and 5 from blood samples drawn at baseline and again at 1, 2, 4, 6, 8, 10, and 12 h after oral opioid administration. Given that the opioids were administered every 12 h, these measurements represent plasma concentration levels at steady state. On day 5, in addition to the opioid pharmacokinetics samples, THC plasma levels were measured at baseline and at 3, 10, 30, and 60 min to determine THC exposure for purposes of comparison with findings of prior and future studies. Our previous studies had demonstrated that this time course encompasses most of the THC AUC.[19]

The main outcome measure was the AUC_{12} for morphine and its glucuronide metabolites, or for oxycodone and its major metabolites, oxymorphone and noroxycodone.

Samples were shipped in a frozen state to the Center for Human Toxicology at the University of Utah, where they were analyzed for cannabinoids, morphine, and oxycodone using published procedures. Briefly, morphine, morphine-3-glucuronide, and morphine-6-glucuronide were measured using liquid chromatography with electrospray ionization–tandem mass spectrometry, with lower limits of quantification of 0.50 and 0.25 ng/ml for morphine and the glucuronides, respectively.[29] Oxycodone, oxymorphone, and noroxycodone were measured using liquid chromatography with electrospray ionization–tandem mass spectrometry, with lower limits of quantification of 0.2 ng/ml for all analytes.[30]

Cannabinoid measurements were obtained using a combination of modifications of previously published methods. The samples underwent liquid–liquid extraction,[31] and both extracts were combined and then derivatized and analyzed as previously described,[32] except that the method was modified to suit a different instrument (i.e., a Hewlett Packard 5890 GC (Palo Alto, CA) equipped with a DB-5 MS, 30 m × 0.25 mm, 0.25-mm column and interfaced with a Finnigan MAT SSQ 7000 MS (San Jose, CA) in negative chemical ionization mode).

Effects monitoring. Objective and subjective effects were measured to assess whether vaporized cannabis increases or attenuates the side effects associated with opioid analgesics. Subjective effects were assessed via participants' self-reports using the Drug Effects Questionnaire administered before the morning opioid dose and again at 30 min and 1, 2, 4, 6, 8, 10, and 12 h after drug administration on days 1 and 5. This questionnaire records subjective findings using standard visual analog scales where 0 is "no effect" and 100 is "maximal effect."[33] Assessment of drug effects included pain, stimulation, anxiety, sedation, feeling "down," hunger, mellowness, confusion, irritation, depression, feeling withdrawn, dizziness, nausea, and dryness of the mouth. In addition, the subjects were evaluated by the nursing staff for side effects every 4 h, recording scores for anxiety, sedation, disorientation, paranoia, confusion, dizziness, nausea, urinary retention, constipation, emesis, headache, swollen extremities, twitching, excitement, and level of consciousness on a scale from 0 to 4. The participants were monitored daily for nausea and vomiting using the Rhodes Index of Nausea, Vomiting, and Retching Questionnaire.[34] Because there was a concern that enhanced opioid effects could lead to respiratory depression, continuous pulse oximetry was performed every night, with the results documented every 2 h on the nursing flowsheet.

Statistical analysis.

Sample size: In a published study of individuals who took morphine on an empty stomach, the standard deviation of the within-person change in log (AUC_{10}) for a morphine solution was 20% over the course of 12 months.[35] Using this information, we estimated that, with a sample of 10 subjects, the study would have 80% power to detect a 25% percent change in the AUC_{12} between days 1 and 5. This estimate was based on a standardized effect size (E/S) of 1.25, using an alpha of 0.05, where E is the within-subject effect size (25%) and S is the standard deviation of the mean of the paired differences (20%) using a paired *t*-test.[36,37] In prior pharmacokinetics studies, a 30% change in AUC was thought to be clinically significant.[38] Therefore, we set the target size at 25% to ensure that we would be able to capture a clinically significant change in $AUC_{12.}$ We enrolled at least 10 participants in each of the two (morphine and oxycodone) groups.

Data analysis: We described the characteristics of the participants at study entry overall and within each opioid group. We presented the mean (with 95% CI) plasma levels for each opioid over the 12-h observation period on days 1 and 5.

The primary outcome was the change in the AUC_{12} for morphine or oxycodone before and after cannabis exposure. We standardized plasma levels for each opioid to doses of 60 mg b.i.d. (observed opioid plasma level × (60 mg/administered opioid dose)). The standardized AUC_{12} was derived using the trapezoidal method over the dosing interval. We estimated the geometric mean and coefficient of variation in

the standardized AUC on days 1 and 5. We then computed the ratio of the geometric means (with 95% CI) for day 5/day 1. We tested the hypothesis of a statistically significant change in standardized AUC_{12} of at least 25%, using paired *t*-tests and nonparametric Wilcoxon signed-rank tests. We also assessed the percentage change in the geometric mean for C_{max} and the arithmetic mean for time to maximum concentration from the plasma concentration-vs.-time data for each subject. We used similar methods to describe results and assess changes for plasma concentrations of the metabolites of morphine (morphine-3-glucuronide and morphine-6-glucuronide) and oxycodone (oxymorphone and noroxycodone). We assessed the mean THC plasma levels (with 95% CIs) for a duration of 1 h, for the participants overall as well as by opioid group.

We described the mean pain ratings on days 1 and 5, both overall and within each opioid group, using mean values and 95% CIs. We assessed the mean values (with 95% CI) of individual differences and percentage changes in pain between days 1 and 5, both overall and within each opioid group, using paired *t*-tests.

Next, we assessed the subjective effects of vaporized marijuana among these participants. We represented the mean perceived high over the dosing period on days 1 and 5 for each opioid group. In addition, we estimated the mean value (with 95% CI) of each subjective effect on days 1 and 5 and determined statistically significant changes in the mean values (with 95% CI) of individual differences, using paired *t*-tests for each opioid group.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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ORIGINAL ARTICLE

Distinct Effects of Δ9-Tetrahydrocannabinol and Cannabidiol on Neural Activation During Emotional Processing

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Context: Cannabis use can both increase and reduce anxiety in humans. The neurophysiological substrates of these effects are unknown.

Objective: To investigate the effects of 2 main psychoactive constituents of *Cannabis sativa* (Δ9-tetrahydrocannabinol [Δ9-THC] and cannabidiol [CBD]) on regional brain function during emotional processing.

Design: Subjects were studied on 3 separate occasions using an event-related functional magnetic resonance imaging paradigm while viewing faces that implicitly elicited different levels of anxiety. Each scanning session was preceded by the ingestion of either 10 mg of Δ9-THC, 600 mg of CBD, or a placebo in a double-blind, randomized, placebo-controlled design.

Participants: Fifteen healthy, English-native, right-handed men who had used cannabis 15 times or less in their life.

Main Outcome Measures: Regional brain activation (blood oxygenation level–dependent response), electrodermal activity (skin conductance response [SCR]), and objective and subjective ratings of anxiety.

Results: Δ9-Tetrahydrocannabinol increased anxiety, as well as levels of intoxication, sedation, and psychotic symptoms, whereas there was a trend for a reduction in anxiety following administration of CBD. The number of SCR fluctuations during the processing of intensely fearful faces increased following administration of Δ9-THC but decreased following administration of CBD. Cannabidiol attenuated the blood oxygenation level–dependent signal in the amygdala and the anterior and posterior cingulate cortex while subjects were processing intensely fearful faces, and its suppression of the amygdalar and anterior cingulate responses was correlated with the concurrent reduction in SCR fluctuations. Δ9-Tetrahydrocannabinol mainly modulated activation in frontal and parietal areas.

Conclusions: Δ9-Tetrahydrocannabinol and CBD had clearly distinct effects on the neural, electrodermal, and symptomatic response to fearful faces. The effects of CBD on activation in limbic and paralimbic regions may contribute to its ability to reduce autonomic arousal and subjective anxiety, whereas the anxiogenic effects of Δ9-THC may be related to effects in other brain regions.

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Ingestion of cannabis can modulate anxiety levels, although the direction of this effect is variable. Long-term cannabis use has been associated with anxiety symptoms,[1-4] panic attacks,[5] and an increased risk of anxiety disorders,[6-8] although the latter remains controversial.[9,10] Acute increases in anxiety can also occur following cannabis use.[11,12] However, cannabis use can also lead to sedation and relaxation, with users often reporting that they take the drug to alleviate psychosocial stress, anxiety, and agoraphobia,[2,13-15] increasing the likelihood of subsequent cannabis abuse.[11,16] Also, patients with psychotic disorders report that they use cannabis to reduce the anxiety associated with psychotic symptoms[17,18] and increased anxiety is a feature of withdrawal from regular cannabis use.[19] There is thus evidence that cannabis can have both anxiogenic and anxiolytic effects. These apparently conflicting observations may partly reflect the fact that *Cannabis sativa* contains multiple compounds that may have different psychoactive properties.[20] In particular, Δ9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD) are the most abundant and both can modulate anxiety. Immediate administration of Δ9-THC can increase anxiety[21] but has also been reported to reduce anxiety and improve sleep.[22,23] This may parallel evidence from studies in experimental animals reporting that low doses of Δ9-THC have anxiolytic effects whereas high doses are anxiogenic.[24-26] In contrast, CBD has anxiolytic effects in both animals and humans,[27-31] and when coadministered with Δ9-THC, it can reduce the anxiety and psychotic symptoms induced

by the latter cannabinoid.[32] These differences in the behavioral effects of Δ9-THC and CBD are paralleled by differences in their mechanism of action at the molecular level. Δ9-Tetrahydrocannabinol binds to neuronal CB1 receptors,[33] which are found on GABAergic and glutamatergic neurons throughout the brain and are thought to be receptors for endogenous anandamide.[34-36] Since CBD has a very low affinity for the cannabinoid CB1 receptor[37,38] and does not bind to benzodiazepine receptors,[29] the molecular mechanisms underlying its anxiolyticlike activity are still unclear. It may activate vanilloid receptors and inhibit the cellular uptake and enzymatic hydrolysis of anandamide,[39,40] activate serotonin $5HT_{1A}$ receptors,[41] and inhibit uptake of adenosine.[42]

Functional neuroimaging provides a sensitive means of examining how cannabis acts on the brain. Although previous neuroimaging studies that have compared long-term cannabis users with healthy controls have demonstrated altered brain activity in prefrontal and cerebellar regions during cognitive tasks,[43] no study has investigated the effect of cannabis on emotional processing. Moreover, comparisons of long-term cannabis users and healthy controls are confounded by demographic, psychiatric, and cognitive differences between these groups, and because cannabis comprises several different psychoactive ingredients, it is unclear which of its constituents are responsible for the findings. The aim of the present study was to use functional magnetic resonance imaging (fMRI) to investigate the neurophysiological basis of the effects of cannabis on anxiety, focusing on Δ9-THC and CBD. We measured the effects of controlled doses of each compound on regional brain activity in healthy volunteers while they were viewing faces with fearful expressions that implicitly provoked anxiety. Subjects were scanned on 3 separate occasions, with each session preceded by ingestion of either Δ9-THC, CBD, or placebo, in a double-blind, randomized, placebo-controlled design. We recorded electrodermal skin conductance responses (SCRs) as a measure of autonomic arousal and assessed the severity of anxiety before, during, and after scanning using subjective and objective instruments. We expected that viewing fearful relative to neutral faces would be associated with activation in a distributed network of areas including extrastriate, prefrontal, cingulated, and medial temporal cortex and the amygdala[44,45] and an altered electrodermal response.[46,47] We tested the hypothesis that CBD would be associated with an attenuation of blood oxygenation level–dependent (BOLD) signal in response to fearful faces in the limbic and paralimbic components of this network (the amygdala and the parahippocampal and cingulate cortex) and an attenuation of the electrodermal response.[30] A further prediction was that these effects would not be evident with Δ9-THC, which if anything, would have effects in the opposite direction.[32]

METHODS

SUBJECTS

Fifteen healthy, English-native, right-handed men (mean [SD] age, 26.67 [5.7] years; age range, 18-35 years) who had a lifetime exposure to cannabis of 15 times or less, with no cannabis use in the last month, no personal or family history of psychiatric illness, and no alcohol or other drug abuse (see later) or dependence were recruited through advertisement in the local media.

Mean (SD) IQ measured using the National Adult Reading Test[48] was 98.67 (7.0). Cannabis and other illicit substance use was assessed using the Addiction Severity Index and drug abuse was defined as "moderate use of small quantities regularly or large amounts occasionally."[49] Participants were requested to abstain from any recreational drug use and medicines for the duration of the study, alcohol intake for 24 hours, and caffeine for 12 hours before each study day. Prior to each session, subjects had urine drug screen analyses for amphetamines, benzodiazepines, cocaine, methamphetamine, opiates, and Δ9-THC using immunometric assay kits. No participants had positive results. The study was approved by the local ethical committee and all participants gave their informed consent.

EXPERIMENTAL DESIGN

Each participant was scanned 3 times with a 1-month interval between scans. After at least 8 hours of fasting, subjects were instructed to have a light standardized breakfast 2 hours before the experiment. Prior to each scanning session, participants were given gelatin capsules of either 10 mg of Δ9-THC or 600 mg of CBD (both approximately 99.6% and 99.9% pure, respectively, and supplied by THC-Pharm, Frankfurt, Germany) or a capsule of placebo (flour).

These were identical in appearance and taste and neither the experimenters nor the participants knew what tablets were being administered in a double-blind procedure. Magnetic resonance imaging (MRI) scans and electrodermal activity (SCRs) were taken between 1 and 2 hours after administration of the drug. Periodic (at baseline and 1, 2, and 3 hours postadministration) psychopathological ratings (mood, Visual Analogue Mood Scale[50] [VAMS]; anxiety, Spielberger State-Trait Anxiety Inventory[51] [STAI]; intoxication, Analogue Intoxication Scale[52] [AIS]; psychotic symptoms, Positive and Negative Syndrome Scale[53] [PANSS]) were collected in all participants. Prior to the experiment each volunteer had performed a training session completing all the scales. Blood samples were taken at the same points from an indwelling intravenous catheter in the nondominant arm of each participant to monitor the levels of drugs (CBD and Δ9-THC as measured in the whole blood by Tricho-Tech, Cardiff, Wales). Heart rate and blood pressure were monitored continuously throughout the procedure. All these procedures were conducted by psychiatrists (P.F.P. and S.B) experienced in the clinical effects of Δ9-THC and CBD who monitored participant well-being during the entire session. No serious adverse events (death, hospitalization, emergency department visit) occurred during the study. Three subjects from the original samples (n = 18) had a psychotic reaction (as assessed by the PANSS and clinical manifestation) to Δ9-THC administration and were excluded since they were unable to perform the tests (final sample, n = 15). These subjects were followed up for 24 hours until the psychotic symptoms relieved. They were further monitored monthly and remained well, with no psychiatric or clinical symptoms.

fMRI PARADIGM

Study subjects participated in one 6-minute experiment using event-related fMRI, where they were presented with 10 different facial identities, each expressing 50% (mildly fearful) or 100% (intensely fearful) intensities of fear or a neutral expression (Facial Expressions of Emotion: Stimuli and Tests).[54] There were thus 30 different facial stimuli in total; each stimulus was presented twice for 2 seconds. Individuals therefore viewed 60 stimuli in total. The order of facial identities and expression

type was pseudorandomized such that there was no successive presentation of the same identity or facial expression type. During the interstimulus interval, the duration of which was varied from 3 seconds to 8 seconds according to a Poisson distribution with an average interval of 5.9 seconds, individuals viewed a fixation cross.[55] They were requested to decide on the gender of face stimuli and press 1 of 2 buttons accordingly. Throughout image acquisition, accuracy and reaction times were monitored via button press and recorded on a PC. Other cognitive paradigms were completed at the same time, the results of which are not reported in this article.

IMAGE ACQUISITION

Images were acquired on a 1.5-T Sigma system (GE Healthcare, Milwaukee, Wisconsin) at the Maudsley Hospital, London, England. T2*-weighted images were acquired with a repetition time of 2 seconds, echo time of 40 milliseconds, and flip angle of 90° in 16 axial planes (7 mm thick), parallel to the anterior commissure–posterior commissure line. A high-resolution inversion recovery image data set was also acquired to facilitate anatomical localization of activation.

IMAGE PROCESSING AND ANALYSIS

Functional MRI data were analyzed with statistical parametric mapping software (SPM5; Wellcome Department of Cognitive Neurology, London) running under the MATLAB7.1 environment. All volumes were realigned to the first volume, corrected for motion artifacts, mean adjusted by proportional scaling, normalized into standard stereotactic space (template provided by the Montreal Neurological Institute), and smoothed using a 6-mm full-width-at-half-maximum gaussian kernel. The time series were high-pass filtered to eliminate low-frequency components (filter width, 128 seconds) and adjusted for systematic differences across trials. The onset times (in seconds) for each trial of neutral, mildly fearful, and intensely fearful faces convolved with a canonical hemodynamic response function. Each task condition (neutral, mildly fearful, and intensely fearful) was then contrasted against the baseline condition (cross fixation) for each of the drug treatments (placebo, CBD, and Δ9-THC). A further comparison contrasted all fearful faces (mildly plus fearful) against neutral faces for each drug treatment (placebo, CBD, and Δ9-THC) to control for activation related to processing faces independent of their emotional expression. To test our hypothesis that there were between-group differences, the activation for each task condition was then compared between drugs, using an analysis of variance within-subjects test. Small-volumes correction (sphere of 12-mm radius) was used for clusters observed in hypothesized regions of interest (limbic and paralimbic areas). Whole-brain voxel-wise threshold was set at $P = .001$, uncorrected, with an extent threshold of more than 20 continuous voxels/clusters.[30] Regional activation was reported at a cluster threshold of $P < .05$ corrected. To investigate the effects of symptom measures (anxiety, intoxication, sedation, positive symptoms) and of the SCR on brain activation, mean change (between 1-2 hours after administration of the drugs, the time when the images were acquired) in the STAI, AIS, VAMS, and PANSS positive symptoms subscale scores and the number of SCR fluctuations (as recorded during the scanning) were, respectively, used as covariates for the contrasts between Δ9-THC/CBD and placebo.

SCR ANALYSIS

Skin conductance was recorded during the fMRI scanning via a pair of silver-silver chloride electrodes with 0.05M sodium chloride gel placed on the distal phalanges of digits II and III of the nondominant hand. The electrode pairs were supplied by a constant voltage and the current change representing conductance was recorded using the DC amplifier. The number, amplitude, and rise time of SCR fluctuations were recorded. A fluctuation was defined by an unambiguous increase (0.01 microsecond) with respect to each pretarget stimulus baseline and occurring 0.5 to 3 seconds after the target face stimulus.[56] The fluctuation amplitude was measured as the difference in skin conductance level from the onset (the skin conductance measure before the first rising data point) to the fluctuation peak. The number and amplitude of SCRs were scored using customized software that allows each SCR to be linked to the individual eliciting stimulus.

STATISTICAL ANALYSIS

SPSS version 15.00 (SPSS Inc, Chicago, Illinois) was used to analyze performance and questionnaire data. Measures of task performance, symptom ratings, physiological data, and drug levels were analyzed using repeated-measures analyses of variance to compare drug conditions. When significant differences were found, using a significance level of 95%, the Tukey test for pairwise comparisons was applied. Using power calculations,[57] we estimated the number of subjects required for detecting significant differences in the amygdala between the placebo condition and the CBD condition with an α (type I error) of .05 and a power of 90%.[30] The anticipated within-group SD was 0.035 and the anticipated minimal difference was 0.037; this resulted in a sample size of 12.

RESULTS

The physiological and behavioral results are based on ratings made at 1 and 2 hours after drug administration, the period during which the fMRI data were acquired.

PHYSIOLOGICAL AND BIOCHEMICAL RESULTS

At 1 and 2 hours after drug administration, the mean (SD) blood levels of Δ9-THC were 3.9 (7.3) and 5.1 (5.6) ng/mL, respectively, and the mean (SD) blood levels of CBD were 4.7 (7) and 17 (29) ng/mL. Compared with placebo, neither Δ9-THC nor CBD significantly affected heart rate or blood pressure at these points ($P < .05$), although we did identify a (nonsignificant) trend for an increase in heart rate with THC: 1.93 (SD 5.74) beats/min and 8.79 (SD 16.31) beats/min at 1 and 2 hours after baseline.

SYMPTOM RATINGS

No significant differences were observed between the drug conditions at baseline for any symptom variable ($P > .05$). Pairwise comparisons revealed that mean anxiety (STAI), intoxication (AIS), sedation (VAMS mental sedation subscale), PANSS positive symptoms subscale (**Figure 1**), PANSS negative symptoms subscale, PANSS general psychopathology subscale, and PANSS total scores (eFigure, http://archgenpsychiatry.com) were significantly increased following Δ9-THC as compared with placebo administration ($P < .05$). Compared with placebo, CBD administration did not significantly change subject rating on any of these measures. However, there was a trend ($P = .06$) for reduction in anxiety following CBD relative to placebo administration on the VAMS anxiety and tranquilization subscale. There was no statistically signifi-

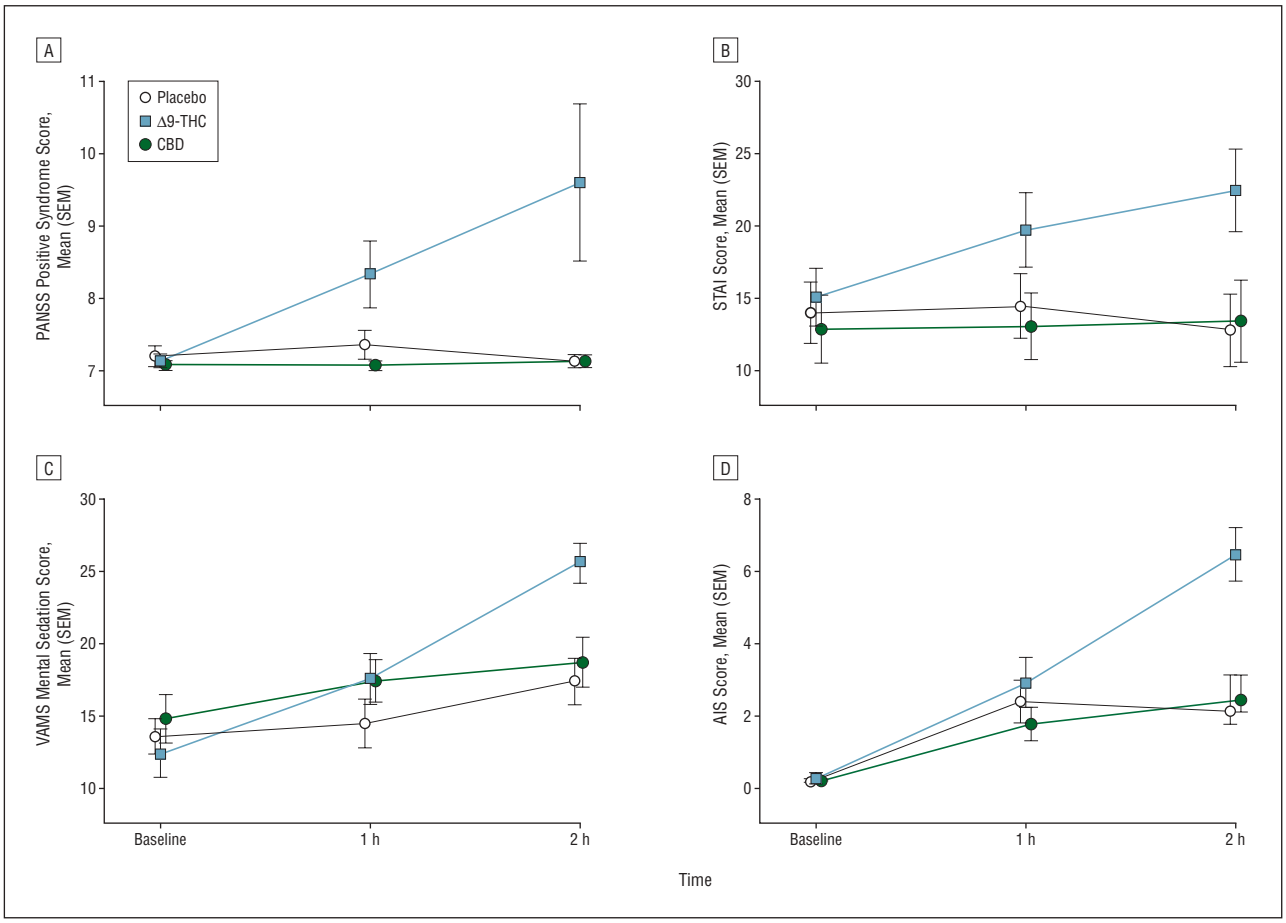

Figure 1. Psychopathological effect of Δ9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD) over time. Plots showing changes in positive symptoms as indexed by Positive and Negative Syndrome Scale (PANSS) positive symptom ratings (A), anxiety as indexed by State-Trait Anxiety Inventory (STAI) ratings (B), sedation as indexed by Visual Analogue Mood Scale (VAMS) mental sedation subscale rating (C), and intoxication as indexed by Analogue Intoxication Scale (AIS) rating (D) under the effect of Δ9-THC, CBD, and placebo over time. Error bars show standard error of the mean.

cant effect of session order or an interaction effect (drug × session order) on behavioral symptoms.

TASK PERFORMANCE

Cannabidiol

Cannabidiol had no significant effect on gender discrimination relative to placebo. Participants were able to distinguish male and female faces with a mean (SD) accuracy of 83.45% (2.63%) following placebo administration and 83.44% (3.16%) following CBD administration ($t=0.10$; $P=.99$). A main effect for valence was present ($F=16.33$; $P<.001$); for both manipulations, accuracy was better for fearful than neutral faces (all t tests $P<.05$). Cannabidiol had no significant effect on reaction times ($F=0.241$; $P=.63$) (**Figure 2**). There was a significant main effect for valence ($F=13.89$; $P<.01$); reaction time was significantly faster when processing intensely fearful faces than processing mildly fearful and neutral faces ($P<.05$). The interaction between valence and drug (placebo/CBD) was nonsignificant ($F=0.79$; $P=.48$).

Δ9-Tetrahydrocannabinol

Δ9-Tetrahydrocannabinol had no effect on the ability of participants to distinguish male and female faces (mean [SD] accuracy of 83.45% [2.63%] following placebo administration and 82.49% [3.86%] following Δ9-THC administration; $t=-1.16$; $P=.27$). There was a significant effect for valence ($F=12.63$; $P=.001$), with better accuracy when processing fearful than neutral faces (all t tests $P<.05$), but no interaction between valence (neutral/mildly fearful/intensely fearful faces) and drug (placebo/Δ9-THC) ($F=0.825$; $P=.46$).

Analysis of reaction times revealed that there was a significant effect for valence ($F=7.56$; $P<.01$) but no significant effect for drug ($F=0.155$; $P=.70$) and no interaction between valence and drug ($F=0.22$; $P=.86$).

SCR RESULTS

SCR Fluctuations

Repeated-measures analyses of the effects of valence (neutral/mildly fearful/intensely fearful) and drug (CBD/placebo/Δ9-THC) revealed main effects of both valence ($F=34.56$; $P<.01$) and drug ($F=23.37$; $P<.01$) on the number of SCR fluctuations and a drug vs valence interaction ($F=7.41$; $P<.05$). Post hoc paired t tests revealed that, compared with placebo, Δ9-THC increased SCR fluctuations during the processing of both intensely and mildly fearful faces but not neutral faces

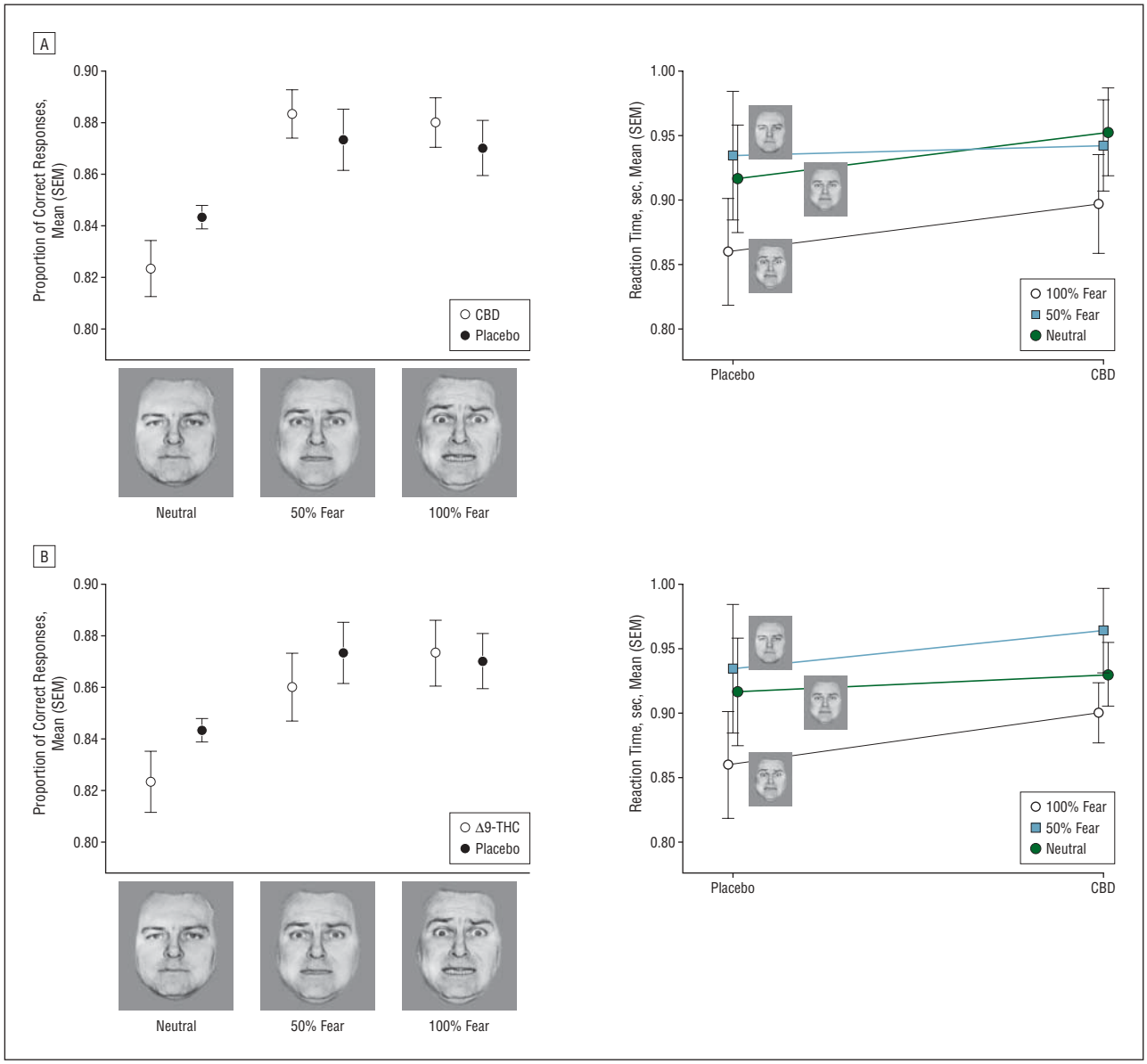

Figure 2. Behavioral effect of Δ9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD). A, Accuracy (left) and reaction time (right) of gender discrimination task across emotional processing (neutral, mildly fearful, intensely fearful) during the placebo or the CBD condition. Error bars show standard error of the mean. B, Accuracy (left) and reaction time (right) of gender discrimination task across emotional processing (neutral, mildly fearful, intensely fearful) during the placebo or the Δ9-THC condition. Error bars show standard error of the mean.

($P<.05$). Conversely, relative to placebo, CBD significantly decreased the number of SCR fluctuations during the processing of intensely fearful, but not mildly fearful or neutral, faces ($P<.05$) (**Figure 3**).

SCR Amplitude

Repeated-measures analysis revealed a main effect of valence on SCR amplitude ($F=4.88$; $P<.05$), with a greater amplitude for intensely fearful than neutral faces ($P<.05$). There was also a main effect of drug ($F=6.75$; $P<.05$) due to Δ9-THC increasing the amplitude of SCR relative to both CBD and placebo ($P<.05$). No significant interaction between drug and valence ($F=0.135$; $P>.05$) was found.

SCR Fluctuation Latency

Neither drug ($F=0.582$; $P>.05$) nor valence ($F=0.506$; $P>.05$) had a significant effect on SCR fluctuation latency.

fMRI RESULTS

Effect of Task (Independent of Drug)

Viewing neutral faces was associated with bilateral activation in the cuneus, fusiform gyrus, inferior occipital gyrus, lingual gyrus, and cerebellum and deactivation in the posterior part of the bilateral superior temporal gyrus. Viewing mildly fearful faces was associated with bilateral activation in the fusiform gyrus, cuneus, lingual

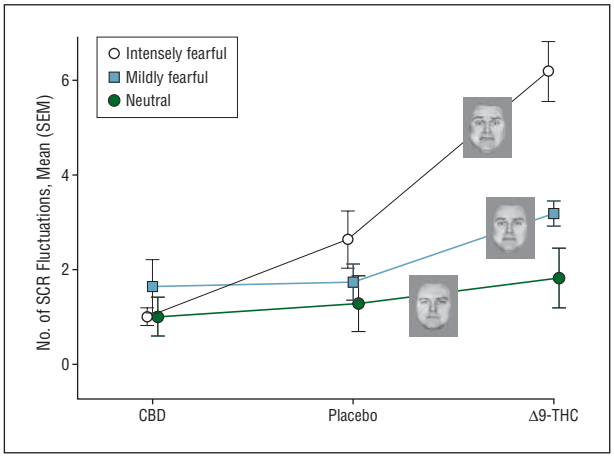

Figure 3. Number of skin conductance response (SCR) fluctuations across psychopharmacological challenge. Error bars show standard error of the mean. CBD indicates cannabidiol; Δ9-THC, Δ9-tetrahydrocannabinol.

gyrus, and cerebellum and in the left parahippocampal, postcentral, and medial frontal gyri. Viewing intensely fearful faces was associated with activation in the left cuneus, the right superior occipital gyrus, the cerebellum bilaterally, the left parahippocampal gyrus and amygdala, the anterior and posterior cingulate cortex, the left inferior and superior parietal lobule, and the right middle frontal, right inferior frontal, and left superior frontal gyri.

Effects of CBD and Δ9-THC on Activation

CBD vs Placebo. Cannabidiol did not significantly affect activation during the processing of neutral faces. During the processing of 50% fearful faces, CBD decreased activation in a region in the posterior lobe of the cerebellum bilaterally (lobule VI) but was not associated with any increases in activation (eTable). The most marked effects of CBD on activation were evident when subjects were processing intensely (100%) fearful faces. Cannabidiol attenuated the BOLD signal in a left medial temporal region, which included the amygdala and the adjacent part of the anterior parahippocampal gyrus, and in the anterior and posterior cingulate gyri, the left middle occipital gyrus, and the right posterior lobe of the cerebellum (**Figure 4**A and eTable). The attenuation of BOLD signal in both the left amygdala and the anterior cingulate significantly covaried with the number of SCR fluctuations while processing 100% fearful faces (Figure 4B). Covarying for AIS and STAI scores had no influence on the effect of CBD on activation in these or any of the other regions.

When the analysis was repeated using neutral faces as the baseline condition rather than visual fixation, CBD decreased activation in the left anterior cingulate, right posterior cingulate, left amygdala, and right cerebellum during the processing of fearful faces (mildly plus intensely fearful), but there was no effect on activation in the occipital cortex (eTable).

Δ9-THC vs Placebo. During the processing of neutral faces, Δ9-THC increased activation in a cluster spanning the posterior-middle temporal gyrus and the left inferior parietal lobule (x=−40, y=−56, z=−24; number of voxels=123, z=6.05) and was not associated with reduced activation in any region. During the processing of mildly fearful faces, Δ9-THC increased activation in the right inferior parietal lobule and was associated with decreased activation in the left medial frontal gyrus (eTable). During the processing of intensely fearful faces, Δ9-THC increased activation in the left precuneus and in the primary sensorimotor cortex bilaterally but decreased activation in the middle frontal gyrus bilaterally and in the posterior cingulate gyrus (**Figure 5** and eTable). Covarying for STAI, PANSS, and AIS scores had no effect on the effect of Δ9-THC on activation in these or any of the other regions. During the processing of fearful faces (mildly plus intensely fearful), Δ9-THC decreased activation in the right inferior frontal gyrus, right superior temporal gyrus, and left medial frontal gyrus and increased activation in the left precuneus (eTable).

COMMENT

The present study used fMRI to investigate the effects of the 2 main psychoactive constituents of *C sativa,* Δ9-THC and CBD, on the neural substrate of emotional processing. To our knowledge, this is the first time neuroimaging has been used to address this issue and the first time the effects of both Δ9-THC and CBD have been assessed in the same subjects.

We used an event-related paradigm with faces that implicitly elicited different levels of anxiety.[55] As expected, processing fearful faces was associated with activation in a network of visual (precuneus, fusiform gyrus, lingual gyrus, cuneus, middle occipital gyrus), limbic (parahippocampal gyrus, amygdala), and paralimbic (posterior and anterior cingulated) regions that mediate the processing of facial emotion.[44] These changes in activation were accompanied by changes in SCR that are typically seen with increased anxiety.[46,47] These neural and electrodermal effects were not attributable to effects of the drugs on performance or attention, as CBD and Δ9-THC did not significantly affect the speed or accuracy of performance on the gender discrimination task. The statistical power of fMRI data has been shown to be relatively robust even with small subject numbers.[58] Functional neuroimaging techniques detect changes at the physiological level and are more sensitive than behavioral measures.[59]

Our main hypothesis was that CBD would attenuate the BOLD response to fearful faces in limbic and paralimbic areas, as well as the accompanying electrodermal response, in line with its anxiolytic effects at the behavioral level.[30] Consistent with this prediction, CBD attenuated BOLD signal in response to intensely fearful faces in the amygdala and the anterior and posterior cingulate cortex, regions that play a crucial role in mediating responses to anxiogenic stimuli (see later). Fearful faces presented as in the present study, in which they alternate with neutral faces, provoke a transient anxious response to each stimulus without necessarily producing a persistent elevation in anxiety.[55] This may explain why CBD did not have a significant effect on the ratings of anxiety during the course of the experiment, consis-

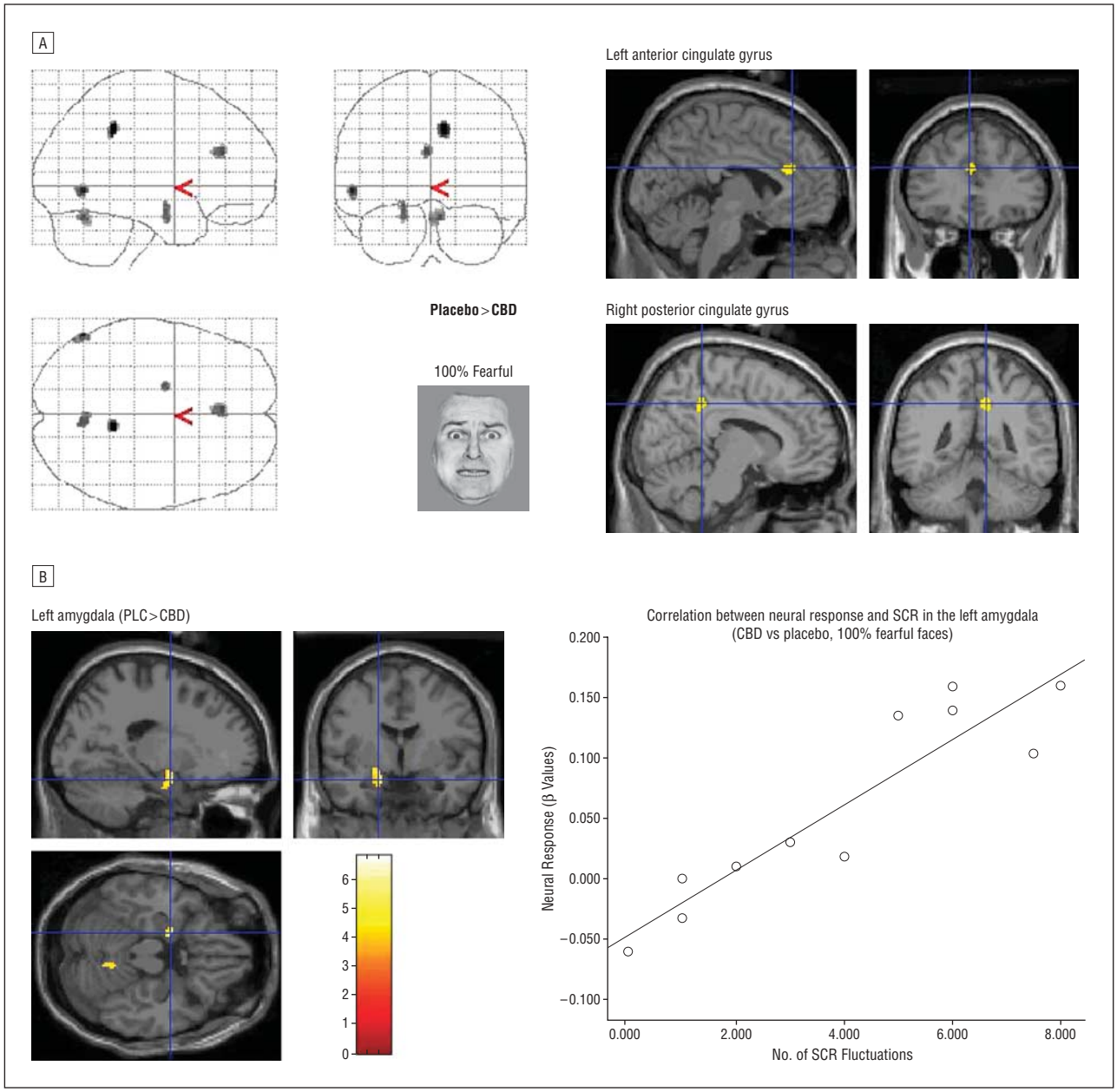

Figure 4. Effect of cannabidiol (CBD) on the brain activation during processing of intensely fearful faces. A, Functional overlay: placebo > CBD. Left side of the Figure is the left side of the brain. B, Correlation between blood oxygenation level–dependent response in the left amygdala during the processing of intensely fearful faces (placebo > CBD) and skin conductance response (SCR).

tent with evidence that CBD can only reduce anxiety if it is already elevated.[20,27,29,31,32,40,60] Nevertheless, there was a trend for reduced subjective anxiety following CBD relative to placebo administration on the VAMS anxiety and tranquilization subscale.

The amygdala is normally activated when subjects are presented with fearful compared with neutral faces,[61-66] and patients with amygdalar lesions are impaired at recognizing fearful faces[67] and show abnormal electrodermal responses.[68,69] Although making gender judgments about faces, the explicit requirement of the task we used, may also activate the amygdala,[70] this was a component of all conditions and is therefore unlikely to have accounted for the effect of CBD relative to placebo. The effect of CBD on activation was significant in the left, but not the right, amygdala. Previous studies suggest that the processing of negative faces preferentially involves the left amygdala,[62,71] while activation in the right amygdala has been associated with processing exaggerated[61] or masked facial expressions of fear,[72] auditory presentations of fear,[64] and aversive tastes.[73] The correlation between the magnitude of the effect of CBD on the amygdalar response to fearful faces and its effect on the electrodermal response to the same stimuli is consistent with evidence that electrical stimulation of the amygdala enhances the SCR in experimental animals[46] and that the SCR during emotional processing in humans is correlated with activity in the amygdala.[46,47,74]

Cannabidiol also modulated the response to fearful faces in the anterior and posterior cingulate cortex. The

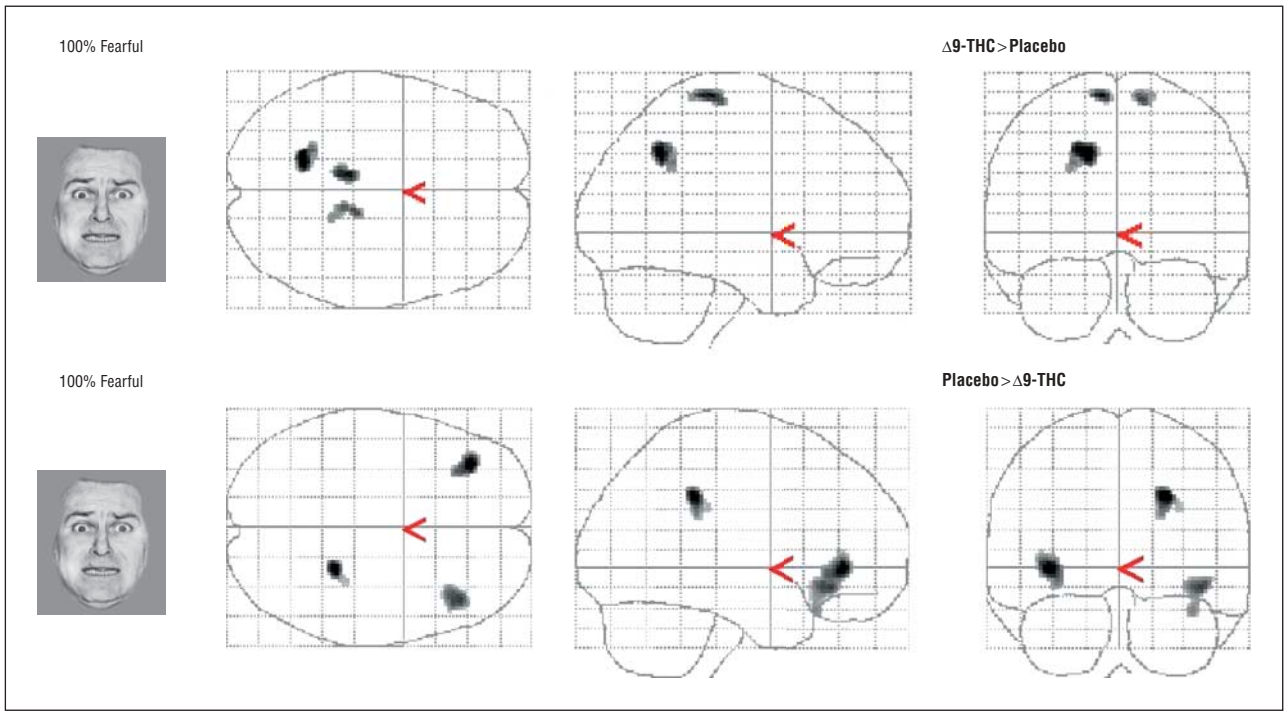

Figure 5. Effect of Δ9-tetrahydrocannabinol (Δ9-THC) on brain activation during processing of intensely fearful faces, glass brain. Functional overlays: Δ9-THC > placebo (top) and placebo > Δ9-THC (bottom). Left side of the Figure is the left side of the brain.

cingulate cortex is critically involved in processing emotional information both in animals[75,76] and in humans.[77] The anterior cingulate cortex is anatomically connected to the amygdala,[78] and neuroimaging studies in humans indicate that the anterior cingulate cortex is engaged with the amygdala in response to fear and anxiety.[79-81] Our findings of effects of CBD in the amygdala and cingulate cortex are consistent with those in the only previous neuroimaging study involving CBD. Using single-photon emission tomography, that study found that CBD modulated resting activity in the left amygdala and the left posterior cingulate among other brain areas, in association with an anxiolytic effect.[30]

Although this was not predicted, we also found that CBD modulated activation in the posterior lobule of the cerebellum (lobule VI) during the processing of fearful faces. There is increasing evidence that the cerebellum plays a role in emotional processing.[82] Patients with lesions in the posterior lobule of the cerebellum (the "cognitive cerebellum") can experience a flattening or blunting of emotions,[83] and cerebellar activation has been observed in lobule VI in response to externally generated emotions such as happiness, sadness, or disgust.[84] This region has also been implicated in conditioned fear,[85,86] which is attenuated by CBD[87] in animal models. Abnormalities in resting cerebellar activity have been reported in neuroimaging studies of regular cannabis users,[88-94] but as this has also been evident in studies involving THC,[87,88,92] it is unclear whether the findings in cannabis users were related to an effect of CBD.

The mechanism of action of CBD at the molecular level is still unclear. Anxiogenic situations may lead to the release of the endogenous cannabinoid anandamide in the amygdala[95]; anandamide may in turn influence emotional states by regulating outputs from the amygdala to other brain regions.[96,97] Cannabidiol inhibits the hydrolysis of anandamide in mouse brain microsomes[39,98,99] and the carrier-mediated cellular uptake of anandamide in mast cells,[39] suggesting that administration of CBD may enhance endogenous anandamide activity. Overall, the production of anandamide by amygdalar activation in response to fear could be part of a negative feedback system that limits anxiety and participates in the control of anxious states, and it has been suggested that anandamide hydrolysis may be a new target for antianxiety drugs.[96]

As predicted, none of the earlier-mentioned effects of CBD on the amygdalar, cingulate, and electrodermal responses to fearful faces were evident following administration of Δ9-THC. Indeed, Δ9-THC had the opposite effect of CBD on the SCR and was associated with an increase in anxiety, rather than an anxiolytic effect.[60] The effects of Δ9-THC on regional activation were largely in a quite different set of brain regions, primarily in the frontal and parietal cortex, and its effects were not correlated with its influence on skin conductance or anxiety. These observations are consistent with data from previous neuroimaging studies using Δ9-THC, which have mainly reported effects on resting activity in frontal and cerebellar regions, as opposed to limbic areas.[43] Δ9-Tetrahydrocannabinol has more extensive symptomatic and cognitive effects than CBD, which extend beyond emotional processing, including the induction of psychotic symptoms,[21,100] impaired memory,[101,102] and motor function.[12] Its effects on regional activation may be more evident in functional imaging studies involving tasks that engage these processes as opposed to emotional processing. Subjective responses to Δ9-THC intoxication vary widely based on

the individual, their prior experience, and expectations,[103] in line with the observation that some subjects of the original sample (20%) developed full-blown paranoia and with the reported large variability of the PANNS positive symptoms subscale scores. Future imaging-genetic studies will address the genetic vulnerability underlying the individual sensitivity to immediate administration of THC.[104,105]

CONCLUSIONS

Cannabidiol and Δ9-THC had distinct modulatory effects on the regional neural response to fearful faces. Cannabidiol attenuated the neurofunctional engagement of the amygdala and cingulate cortex when subjects viewed intensely fearful stimuli and this effect was correlated with a reduction in the electrodermal response, consistent with behavioral evidence that it has anxiolytic effects. In contrast, Δ9-THC modulated activation in frontal and parietal areas and was associated with an increase in anxiety and the electrodermal response.

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Additional Information: The eFigure and eTable are available at http://www.archgenpsychiatry.com.

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Functional Interactions between Endocannabinoid and CCK Neurotransmitter Systems May Be Critical for Extinction Learning

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The endocannabinoid system and the cannabinoid type 1 receptor (CB1R) are required for the extinction of conditioned fear. CB1 antagonists have been shown to prevent extinction when delivered both systemically and within the amygdala. Anatomical studies suggest that CB1Rs in the basolateral amygdala (BLA) are expressed on GABAergic interneurons expressing the anxiogenic peptide cholecystokinin (CCK). Pre-synaptic CB1Rs inhibit neurotransmitter release, suggesting that CB1R activation during extinction may decrease CCK peptide release as well as GABA release. Thus, we examined whether extinction involves the CB1R modulation of CCK2 receptor activation. We found that intracerebroventricular administration of the CCK2 agonist pentagastrin dose-dependently impaired extinction of conditioned fear. Systemic administration of a CB1 antagonist, rimonabant (SR141716), also potently inhibited extinction learning. This effect was ameliorated with systemic administration of a CCK2 antagonist, CR2945. Furthermore, the extinction blockade by systemic SR141716 was reversed with intra-BLA, but not intrastriatal, infusion of CR2945. Lastly, as extinction usually leads to an increase in Akt phosphorylation, a biochemical effect antagonized by systemic CB1 antagonist treatment, we examined whether CR2945 co-administration would increase extinction-induced p-Akt levels. We observed that extinction-trained animals showed increased Akt phosphorylation following extinction, CB1 antagonist-treated animals showed p-Akt levels similar to those of non-extinction trained animals, and co-administration of CR2945 with SR141716 led to levels of p-Akt similar to those of vehicle-treated, extinction-trained controls. Together, these data suggest that interactions between the endocannabinoid and CCKergic transmitter systems may underlie the process of extinction of conditioned fear.

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INTRODUCTION

Over the past 15 years, the endogenous cannabinoid system and the cannabinoid type 1 receptor (CB1R) have been linked to a staggering array of normal and pathologic functions of the CNS, ranging from excitotoxicity to nociception. Among the most striking behavioral findings regarding the cannabinoid system has been that genetic or pharmacologic antagonism of the CB1R leads to profound deficits in the extinction of conditioned fear (Marsicano *et al*, 2002). This systemic effect of CB1 antagonists has now been demonstrated to occur when an antagonist is delivered locally within the basolateral amygdala (BLA) (Roche *et al*, 2007). Furthermore, we have previously demonstrated that a cannabinoid reuptake inhibitor enhances extinction learning (Chhatwal *et al*, 2005). As disruptions in extinction learning are thought to be major obstacles in the treatment of a variety of psychiatric illnesses, including PTSD, specific phobias, and many anxiety disorders, the endogenous cannabinoid system has become a major therapeutic target in the treatment of fear and anxiety.

Anatomical studies of the CB1R in the CNS have demonstrated that they are often pre-synaptically located, where they are thought to be activated by retrograde diffusion of endocannabinoid (eCB) transmitters. Once activated, CB1Rs act to decrease the excitability of the pre-synaptic terminal, leading to decreases in neurotransmitter release.

High concentrations of CB1Rs have been observed on the pre-synaptic terminals of GABAergic interneurons expressing the anxiogenic neuropeptide cholecystokinin (CCK) in many brain regions (Katona *et al*, 1999; Marsicano and Lutz, 1999; McDonald and Mascagni, 2001). Several

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electrophysiological studies have established that CB1R activation leads to decreases in GABA release and likely CCK release from interneurons in the hippocampus that contain both CCK peptide and CB1R (Katona *et al*, 1999; Beinfeld and Connolly, 2001; Burdyga *et al*, 2004; Fride, 2005).

With respect to extinction, Marsicano *et al* (2002) have demonstrated that re-exposure to conditioned cues in the absence of the original aversive unconditioned stimulus (ie extinction training) is a potent signal for the production of two major eCBs in the amygdala (Marsicano *et al*, 2002). This coupled with the known electrophysiological effects of CB1R activation suggests that activity-dependent reductions in neurotransmitter release from CCK + /CB1 + neurons in the amygdala may play a role in the neurobiology of extinction learning.

Administration of exogenous CCK peptide (usually given as a sulfated version of the terminal 4–8 peptides) to rodents and humans appears to be anxiogenic and, in some cases, panicogenic (Harro *et al*, 1993; Belcheva *et al*, 1994; Vasar *et al*, 1994; Bradwejn and Koszycki, 2001). The anxiogenic effects of CCK peptide agonists are believed to be mediated by the CCK2 (or CCK-B) receptor, a G-protein-coupled receptor expressed widely in the brain. There has also been considerable evidence that CCK2 receptors within the BLA are responsible for these anxiogenic effects (Josselyn *et al*, 1995; Frankland *et al*, 1996). Notably, Frankland *et al* (1996) have shown that intracerebroventricular (i.c.v.) or intra-amygdala administration of the CCK2 agonist pentagastrin leads to increased expression of baseline startle, suggesting that CCK2 receptors within the amygdala are important mediators of fear responses in addition to their role in unconditioned anxiety responses. Furthermore, the same authors have been able to show that administration of a CCK2 antagonist can lead to a decrease in the conditioned, fear-potentiated startle (FPS) response in the rat (Josselyn *et al*, 1995); this finding has been supported by more recent studies showing that two other CCK2 antagonists reduce post fear-conditioning freezing in mice, both with respect to contextual fear conditioning and conditioning to a discrete conditioned stimulus (CS; Izumi *et al*, 1996; Tsutsumi *et al*, 1999).

In this study, we examine the interaction between cannabinoid neurotransmission and CCKergic neurotransmission in the extinction of conditioned fear. Specifically, as the anatomy of the CB1 system suggests that it may be involved in producing activity-dependent, dynamic reductions in CCK and GABA release, we address the hypothesis that the CB1 antagonist-induced blockade of extinction learning may be mediated in part by an inability of eCBs to reduce CCK2 receptor activation during extinction.

MATERIALS AND METHODS

Animals

The procedures used were approved by the Institutional Animal Care and Use Committee of Emory University and in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Adult male Sprague–Dawley rats (Charles River, Raleigh, NC) weighing 350–500 g were used. Animals were housed in pairs in a temperature-controlled (24°C) animal colony, with *ad libitum* access to food and water. They were maintained on a 12 h light/dark cycle with lights on at 0800, with all behavioral procedures performed during the rats' light cycle.

Surgery

In studies utilizing i.c.v. drug administration, 22-gauge stainless-steel guide cannulae were implanted under ketamine/xylazine anesthesia, and secured using dental cement (coordinates: AP: 0, ML: −1.6, DV: −5.0; nosebar: +5.0). Habituation to the testing context and subsequent behavioral testing began 7–10 days following surgery. Similar procedures were used to implant bilateral cannulae aimed at the basolateral complex of the amygdala (22-gauge cannulae, AP: −3.1, ML: ±5.4, DV: −8.4; nosebar: −3.6). Additional control experiments were performed with cannulae aimed at the striatum (22-gauge cannulae, AP: −1.0, ML: ±4.0, DV: 5.0; nosebar: −3.6). Following behavioral testing, cannulated animals were killed and cannula placement was assessed on cryostat-sectioned tissue. Animals with amygdala-placed (or striatally placed) cannulae were included for analysis ($n = 8$ each for vehicle and CR2945-treated groups).

In Situ Hybridization

In situ hybridization was performed as described previously (Ressler *et al*, 2002). A cDNA clone containing the coding sequence of the rat cannabinoid receptor type 1 (IMAGE expressed sequence tag clone, GI accession no. 11375084) and pre-pro CCK (IMAGE expressed sequence tag clone, GI accession no. 4059800) were linearized after sequence verification. Antisense riboprobes were generated with T3 RNA polymerase. Slide-mounted sections of snap-frozen rodent brain tissue were post-fixed, proteinase K digested, and blocked followed by overnight hybridization of the tissue at 52°C with [^{35}S]UTP-labeled riboprobes. After a stringent wash protocol, slides were apposed to autoradiography film.

Two-color fluorescent *in situ* hybridization was performed as described previously (Vosshall *et al*, 1999). In brief, digoxigenin (CCK) or fluorescein (CB1) was used to label CCK and CB1 riboprobes, respectively (Roche Diagnostics, Indianapolis, IN). Hybridization and wash protocols identical to those described above for radiolabeled probes were used. Following quenching of endogenous peroxidases and blocking (30 min in 1% bovine serum albumin diluted in 100 mM Tris-HCl, 150 mM NaCl), hybridized slides were incubated with peroxidase-tagged antibodies against fluorescein (1 : 500 dilution in 1% BSA, 100 mM Tris-HCl, 150 mM NaCl; Roche Diagnostics). Amplification was then performed using an FITC-tyramide conjugate (1 : 50; Perkin-Elmer, Wellesley, MA). Following quenching of the peroxidase activity from the first probe, similar procedures were employed to visualize the digoxigenin-labeled probe (CCK) via the use of a peroxidase-labeled antibody raised against digoxigenin (Roche Diagnostics) followed by amplification with a CY5-tyramide conjugate (Perkin-Elmer).

Startle Apparatus

Animals were trained and tested in $8 \times 15 \times 15$ cm Plexiglas and wire-mesh cages, with floors consisting of four 6.0-mm-diameter stainless-steel bars spaced 18 mm apart. Each cage was suspended between compression springs within a steel frame and located within a custom-designed $90 \times 70 \times 70$ cm ventilated sound-attenuating chamber. Background noise (60-dB wide-band) was provided by a type 1390-B noise generator (ACO Pacific Inc., Belmont, CA) and delivered through high-frequency speakers (Radio Shack Supertweeter; Tandy, Fort Worth, TX) located 5 cm from the front of each cage. Sound level measurements (sound pressure level) were made with a Bruel & Kjaer (Marlborough, MA) model 2235 sound-level meter (A scale; random input) with the microphone (type 4176) located 7 cm from the center of the speaker (approximating the distance of the rat's ear from the speaker). Startle responses were evoked by 50-ms, 95-dB white-noise bursts generated by a Macintosh G3 computer soundfile (0–22 kHz), amplified by a Radio Shack amplifier (100 W; model MPA-200; Tandy), and delivered through the same speakers used to provide background noise. An accelerometer (model U321AO2; PCB Piezotronics, Depew, NY) affixed to the bottom of each cage produced a voltage output proportional to the velocity of cage movement. This output was amplified (model 483B21; PCB Piezotronics) and digitized on a scale of 0–2500 U by an InstruNET device (model 100B; GW Instruments, Somerville, MA) interfaced to a Macintosh G3 computer. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 200 ms after the onset of the startle-eliciting stimulus. The CS was a 3.7-s light (82 lux) produced by an 8 W fluorescent bulb (100 μs rise time) located 10 cm behind each cage. Luminosity was measured using a VWR light meter (Atlanta, GA). The US was a 0.5-s shock, delivered to the floorbars and produced by a shock generator (SGS-004; Lehigh Valley, Beltsville, MD). Shock intensities (measured as in Cassella and Davis, 1986) were 0.4 mA. The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads Inc., Newton, CT). Animals were pre-exposed to the chambers for 10 min on each of 2 days before training to habituate them to handling and the test chambers and to minimize the effects of contextual conditioning.

Fear Conditioning

On 2 consecutive days following habituation, rats were returned to the same chambers and presented with 10 pairings of a light (3.7 s) co-terminating with a 0.4-mA, 0.5-s shock (3.6-min intertrial interval).

Post-training Matching

Twenty-four hours following the last fear-conditioning session, animals were returned to the same chambers and presented with startle stimuli (50-ms, 95-dB white-noise bursts) in the presence or absence of the light-CS (15 light–startle compounds and 10 startle-alone trials). Increased startle in the presence of the light-CS was taken as a measure of conditioned fear, and the magnitude of the fear response was calculated as the percentage by which startle increased when the light-CS was presented along with the startle stimulus *vs* when it was omitted (FPS). Using these measurements, animals were divided into groups displaying approximately equal levels of FPS before drug treatment and extinction training.

Extinction Training (Retention Studies)

Five days following the last fear-conditioning trial, animals were injected intraperitoneally (i.p.) with a test compound or its vehicle in 1 ml/kg volumes and then immediately returned to the same chambers and presented with 90 presentations of the light-CS in the absence of footshock (3.7-s light, 30-s intertrial interval). Light presentations were preceded by 10 95-dB noise burst-alone trials with 30 s ITI to assess potential drug-induced alterations of baseline startle. At 48 and 96 h post-extinction training, animals were tested for the presence of FPS (15 light–startle compounds and 15 startle-alone trials).

Extinction Training (Within-Session Extinction)

In the final experiment in this set of studies, the extinction training program was altered to allow for the assessment of reductions in conditioned fear during the extinction training sessions (within-session extinction). To do this, extinction training was altered to 30 light–startle and 30 startle-alone trials (3.7-s light, 95-dB startle, 30-s interstimulus interval) rather than 90 light-alone trials. FPS was calculated as in the other testing sessions, and results were grouped into 10 blocks of three trials each. To allow for direct comparison of within-session extinction across groups, FPS values were normalized such that each animal's fear response during the first block of trials was considered 100%, a normalization that compensated for variations in FPS before extinction.

Assessment of Drug Effects on Baseline Startle and Unconditioned Fear

A group of behaviorally naïve animals were handled and habituated to the training and testing chambers for 2 consecutive days and then presented with a 15-trial test for the presence of unconditioned fear to the light (15 light–startle compounds and 15 startle-alone trials) and matched into four groups showing equivalent levels of unconditioned fear to the light and equivalent levels of baseline startle. Unconditioned fear to the light was observed to be quite low ($<10\%$ per matched group). Three days following this matching test, animals were injected with vehicle (100% DMSO), SR141716A (5 mg/kg), CR2945 (3 mg/kg), or a combination of SR141716A and CR2945 (5 and 3 mg/kg, respectively). In all cases, drugs were administered in volumes of 1 ml/kg. Twenty minutes following injection, animals were tested for alterations in baseline startle and unconditioned fear to light (5 light–startle compounds and 45 startle-alone trials), followed by two light–shock pairings to assess alterations in shock reactivity.

Drugs

SR141716A (Rimonabant NIMH Drug Supply Program, Bethesda, MD) and CR2945 (Sigma-Aldrich, St Louis, MO) were dissolved in 100% DMSO. A 25 mg portion of pentagastrin (Sigma-Aldrich) was first dissolved in 2.5 ml of 100% DMSO and then serially diluted to generate 100 and 500 nM working solutions. I.c.v. infusions were performed using a flow rate of 1.0 μl/min with a total infused volume of 5 μl. For local infusion of CR2945, a 1 mg/ml solution of CR2945 in 100% DMSO was diluted to generate a working solution of 2 μg/μl CR2945 in 5% DMSO/95% sterile PBS. Intra-BLA infusions were performed using a flow rate of 0.1 μl/min with a total infused volume of 0.5 μl per side (1 μg drug/side).

Western Blotting

Following extinction training, animals were killed using overdoses of isoflurane. Brains were blocked rapidly over ice and dissected into 2-mm-thick coronal sections. The BLA was removed bilaterally using a brain punch tool, and punches from each side were pooled and homogenized in buffer (5 mM HEPES, 0.32 M sucrose, protease inhibitors) and kept frozen at −80°C until western blot assay. Whole-cell lysed samples were tested for protein concentration using a BCA assay (Pierce Biotechnology, Rockford, IL). Fifteen micrograms of protein per animal was loaded onto polyacrylamide-SDS mini-gels, separated electrophoretically, blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA), and blocked for 1 h in 2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, and 10 mM HEPES (pH 7.4) (NDM-HEPES). Membranes were incubated overnight at 4°C in a 1 : 1000 dilution of rabbit × phospho-Akt (Ser473) antibody (Cell Signaling, Danver, MA, no. 9271) in NDM-HEPES buffer and then incubated in a 1 : 2000 dilution of an HRP-labeled secondary antibody for 60 min. The bound antibody was detected by SuperSignal West Chemiluminescence (Pierce Biotechnology) in an Alpha Innotech Fluorchem imaging system (Alpha Innotech, San Leandro, CA). Total blotted protein levels were assessed using levels of α-tubulin (1 : 5000; Sigma), the detection of which was used to control for variations in protein loading.

Statistical Analysis

Comparisons were made across drug treatment groups at each test using an ANOVA or Student's *t*-test with drug or dose as the independent measure. In experiments involving multiple days of extinction training and subsequent testing, a repeated measures ANOVA was used to assess differences between drug groups and to assess drug treatment interactions with time and FPS. In both cases, Fisher's LSD was used for *post hoc* analysis.

RESULTS

CCK mRNA is Coexpressed with CB1 mRNA within the BLA

In situ hybridization was used to determine the patterns of CCK mRNA expression within the rat amygdala and to assess differential expression of CCK mRNA in the basolateral, medial, and central amygdaloid nuclei. In close agreement with previous studies, we observed that both CB1R (Figure 1a) and CCK (Figure 1b) mRNAs were highly

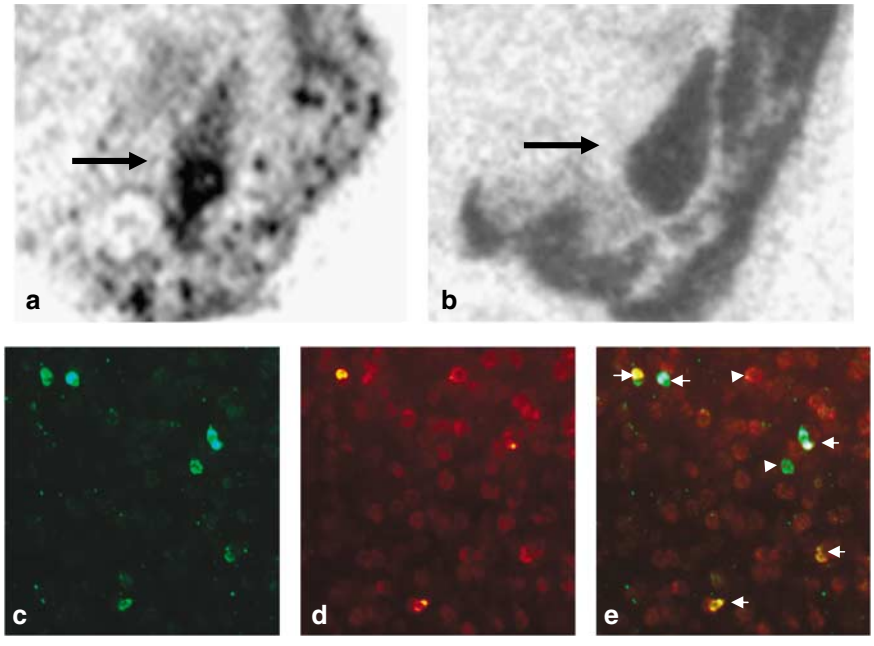

Figure 1 CB1 and CCK mRNAs are highly expressed within the amygdala, and show a high degree of colocalization: the mRNAs for the CB1R (a) and pro-CCK peptide (b) are highly enriched in the basolateral complex of the amygdala (BLA), as compared to the central and medial nuclei of the amygdala, as shown by radiolabeled riboprobes. Using fluorescently labeled riboprobes, the mRNAs for CB1 and CCK were observed to be coexpressed in many of the same cells (arrows), as depicted in a double-labeled section of the BLA shown in panels c (CB1—green), d (CCK—red), and e (overlay). A smaller subset of neurons were observed to be expressing primarily only CB1 or CCK (arrowheads).

expressed in the basal, lateral, and basolateral nuclei of the amygdala (collectively referred to as the basolateral complex of the amygdala (BLA)), whereas much lower levels of both mRNAs were observed in the central and medial amygdala. Using fluorescently labeled riboprobes, we examined whether CB1 and CCK mRNAs were expressed in the same neuronal cells within the BLA. In agreement with previous results (Marsicano and Lutz, 1999; McDonald and Mascagni, 2001), we found that many CB1-expressing cells within the BLA also expressed the mRNA for CCK (Figure 1c and d, arrows), although occasionally cells were observed to express only one of these mRNAs (Figure 1c and d, arrowheads). Notably, double-labeling for CCK and CB1 mRNA expression has not been reported in rats. Marsicano and Lutz (1999) found an overlap of CCK mRNA expression in CB1 mRNA-expressing neurons in the mouse ranging from 47 to 100% depending on the brain region and the level of CB1 expression. In this rat sample, we found that approximately 70% of neurons expressing high levels of CB1 mRNA also express CCK mRNA, consistent with reports in mice.

Pentagastrin, a CCK2 Agonist, Impairs Extinction, Similar to a CB1 Antagonist

We hypothesized that CB1R activation, which is critical for extinction, acts through reduction in CCK release and results in subsequent reduction in CCK2 receptor activation. According to this hypothesis, increasing CCK2 activation by administering a CCK2 agonist should impair extinction. To test this hypothesis, rats were implanted with i.c.v. cannulae, allowed to recover for 7–10 days, fear-conditioned as outlined in Figure 2a, and matched into groups demonstrating similar levels of FPS before extinction. Thirty minutes before extinction training (90 lights without shocks), animals were infused with vehicle, 100 nM pentagastrin, or 500 nM pentagastrin (5 μl/infusion).

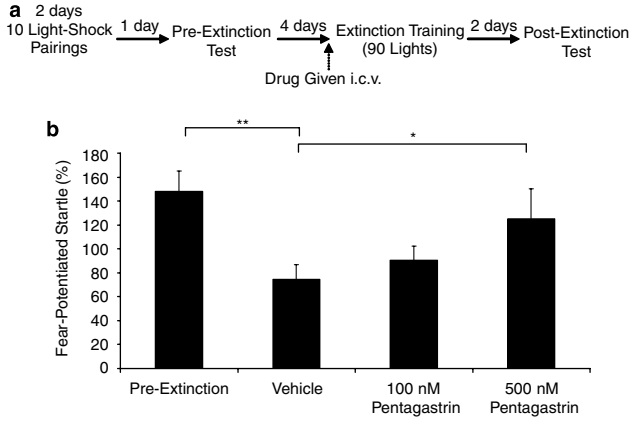

Figure 2 Pentagastrin, a CCK receptor agonist, impairs extinction. Animals were implanted with i.c.v. cannulae 7–10 days before behavioral training (a). Thirty minutes before extinction training, animals received 5 ml infusions of 0, 100, or 500 nM pentagastrin. Animals treated with pentagastrin showed higher levels of fear than vehicle-treated controls when tested off-drug, 48-h following extinction (b) $n = 25$ for vehicle group, $n = 8$ for 100 nM pentagastrin group, $n = 17$ for 500 nM pentagastrin group; values shown are the averages of all test trials; error bars indicate ± SEM; * denotes $p < 0.05$, ** denotes $p < 0.01$.

Two days following extinction training, animals were tested, in the absence of any drug, for the presence of FPS as a measure of conditioned fear. Vehicle-treated control animals showed the lowest levels of FPS following extinction, indicative of significant extinction. In contrast, animals receiving 500 nM pentagastrin at the time of extinction training showed significantly higher levels of conditioned fear than vehicle-treated controls. In animals receiving 100 nM pentagastrin, the levels of FPS were intermediate between vehicle- and 500 nM pentagastrin-treated animals, suggesting that the impairment of extinction retention with pentagastrin may be dose-dependent (Figure 2b; linear contrast ANOVA $F_{(1, 50)} = 5.074$; *post hoc* 500 nM *vs* vehicle, $p < 0.05$). Notably, baseline startle in our training and testing paradigm was not significantly different in pentagastrin-treated animals as compared with controls, either immediately after pentagastrin administrations (Supplementary Figure 1A) or 48 h post-extinction (Supplementary Figure 1B).

Blockade of Extinction by a CB1 Antagonist is Reversed with a Systemic CCK2 Antagonist

It has previously been shown that a cannabinoid antagonist (SR141716A) prevents the normal extinction of conditioned fear when delivered systemically (Marsicano *et al*, 2002; Suzuki *et al*, 2004; Chhatwal *et al*, 2005) and through intra-BLA infusions (Roche *et al*, 2007). Activation of CB1Rs is thought to inhibit the release of GABA and CCK by decreasing the excitability of the pre-synaptic terminal. If preventing CCK release was a critical component of CB1-mediated effects on extinction, we predicted that antagonizing the CCK2 receptor would reverse the normal blockade of extinction seen with CB1R antagonists. Thus, we examined whether co-administration of a CCK2 antagonist (CR2945) might reverse the blockade of extinction seen with CB1 antagonist (SR141716A) treatment. In this series of experiments, animals were again fear-conditioned and matched into groups showing similar levels of FPS before extinction training (Figure 3a). Thirty minutes before extinction training (90 lights without shocks), animals were systemically administered vehicle (100% DMSO), SR141716A (5 mg/kg), CR2945 (3 mg/kg), or a combination of SR141716A and CR2945 (5 and 3 mg/kg, i.p., respectively).

In agreement with previous studies (Marsicano *et al*, 2002; Suzuki *et al*, 2004; Chhatwal *et al*, 2005), we observed that administration of SR141716A potently inhibited extinction learning, as animals receiving SR141716A showed higher levels of FPS than vehicle-treated controls when tested 48- and 96-h post-extinction (Figure 3b). Notably, rats treated with a combination of SR141716A and CR2945 showed significantly less fear than those receiving SR141716A alone and levels of FPS that were statistically similar to those of vehicle-treated controls (Figure 3b; first test $F_{(3, 24)} = 3.876$, $p < 0.05$; second test $F_{(3, 24)} = 3.060$, $p < 0.05$). Rats that received CR2945 alone before extinction training did not show enhanced extinction retention as compared to vehicle-treated animals.

Thus, CCK2 activation is an important downstream effect of CB1R blockade on extinction. Additionally, as we find that in the absence of CB1 blockade, CCK2 antagonism is

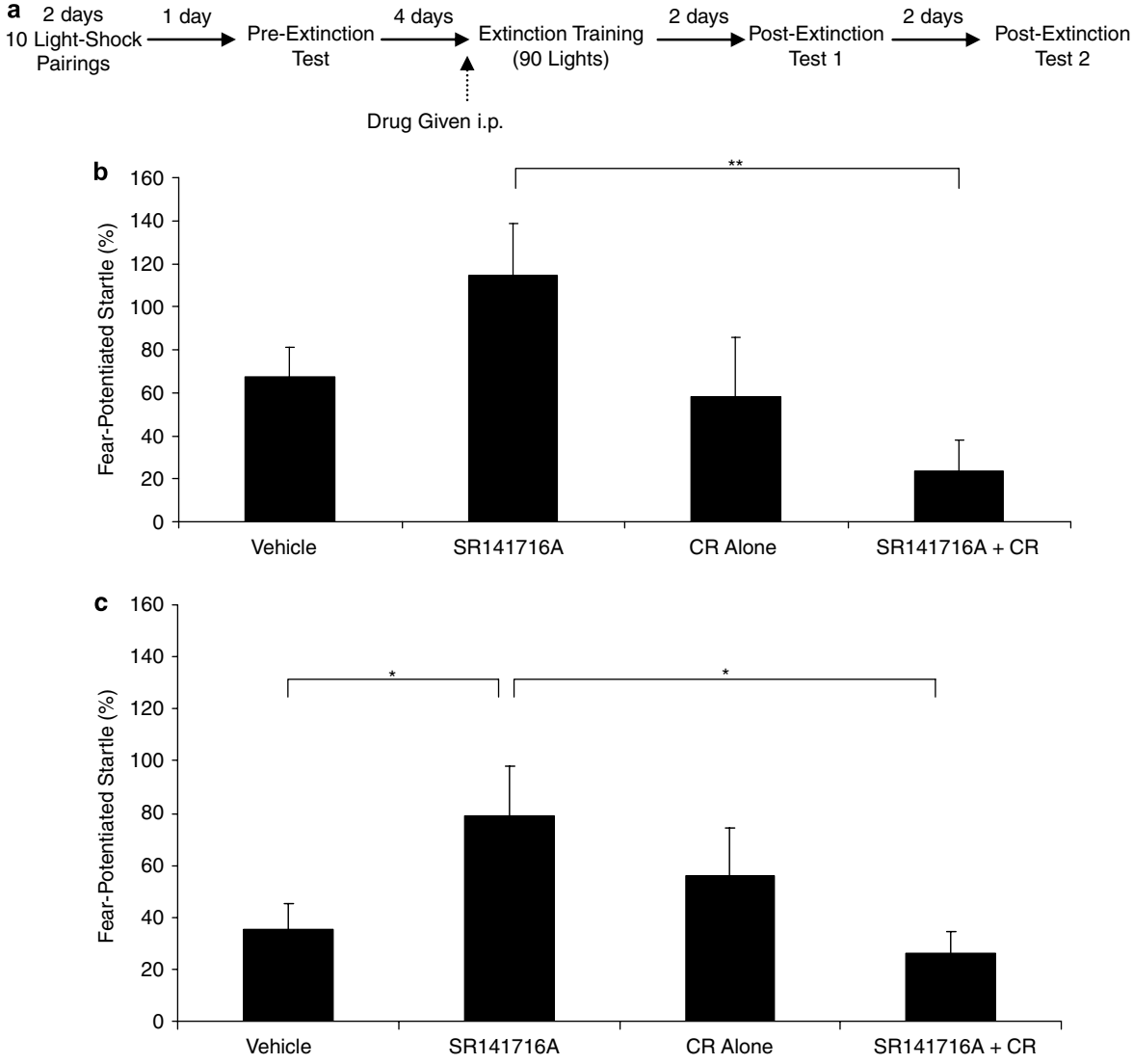

Figure 3 Systemic administration of a CCKB antagonist reverses the blockade of extinction normally seen with CB1 antagonist treatment. Schematic representation of the behavioral paradigm used in these studies (a). Consistent with previous results, we observed that pre-extinction training administration of the CB1 antagonist SR141716a (5 mg/kg, i.p.) produced a profound blockade of extinction retention as measured 48 h (b) and 96 h (c) following extinction training. Vehicle-treated animals and animals co-administered the CCK2 antagonist CR2945 (CR, 3 mg/kg, i.p.) showed significantly less FPS 48 h (b, SR141716A + CR group) and 96 h (c, vehicle and SR141716A + CR group) following extinction training as compared to those receiving SR alone ($n = 7$ per group; values shown are averages of all trials in each test; error bars indicate ± SEM; * denotes $p < 0.05$, ** denotes $p < 0.01$).

not sufficient to enhance extinction, other pathways (eg modulation of GABA release) are likely important as well in extinction modulation at baseline.

Blockade of Extinction by a CB1 Antagonist is Reversed with an Intra-amygdalar CCK2 Antagonist

The BLA is known to be a critical site for extinction learning, and intra-BLA infusions of SR141716A have been shown to prevent extinction of fear (Roche *et al*, 2007). Here, we examined whether the local infusions of CR2945 into the amygdala would mitigate the blockade of extinction seen with systemic SR141716A administration. In these experiments, rats were implanted with bilateral cannulae aimed at the BLA and allowed to recover for 7–10 days. Subsequently, these animals were fear-conditioned and matched into groups showing equivalent levels of startle, as in the aforementioned experiments. Thirty minutes before extinction training (90 lights without shocks), all animals were given i.p. injections of SR141716A (5 mg/kg) along with bilateral infusions of either vehicle (5% DMSO in PBS) or CR2945 (1 μg/0.5 μl/side).

Following extinction training, animals were tested for the presence of FPS 2 days following training. As the animals in these experiments showed relatively little extinction at the first post-extinction test, perhaps as a result of the stress involved in local amygdala infusions, two additional blocks of extinction training (with similar drug treatment) and testing were given (Figure 4a). Importantly, we focused on the amount of FPS demonstrated in the first five trials of each post-extinction testing session and used this to assess extinction retention, as a great deal of within-session

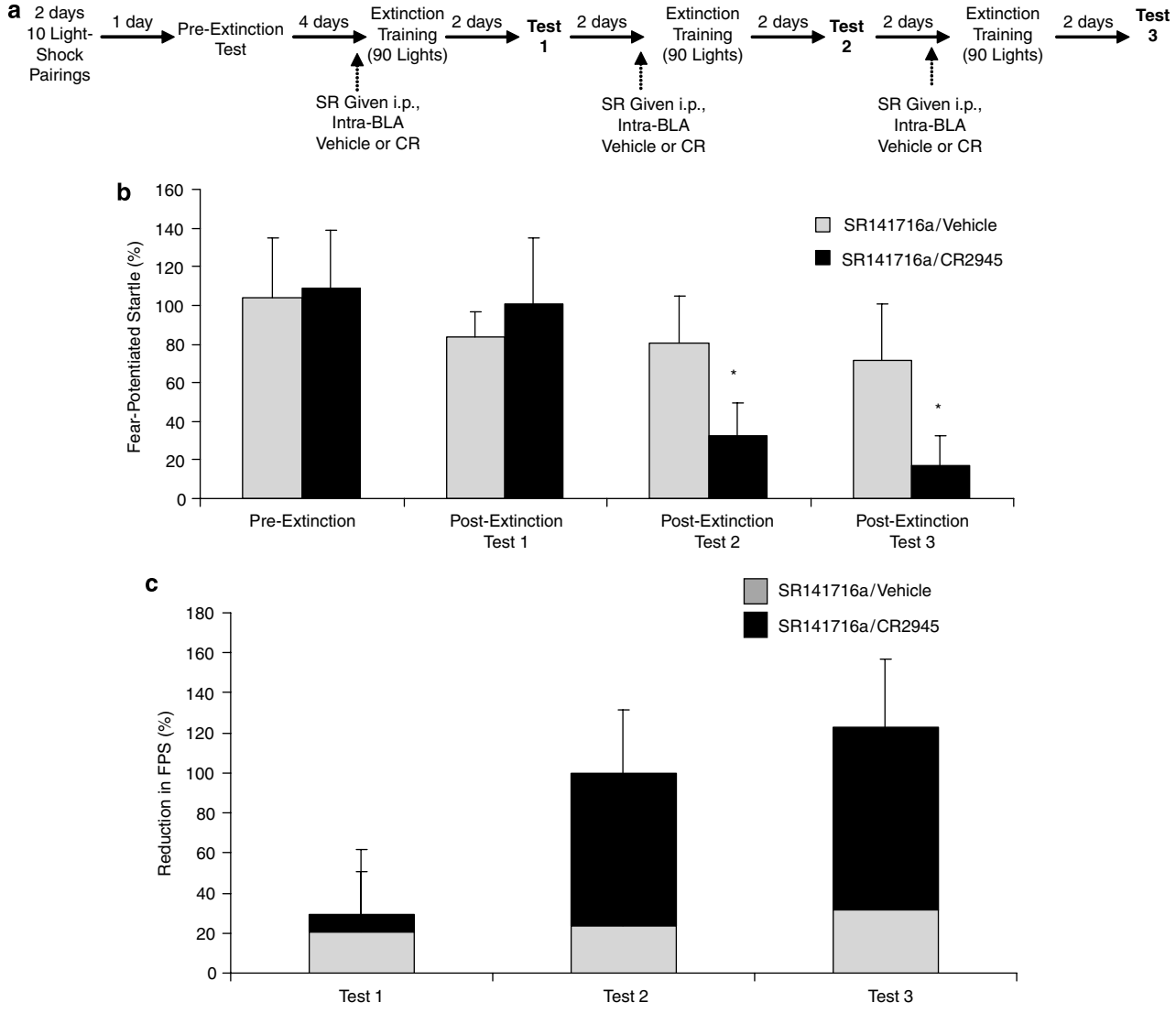

Figure 4 Intra-amygdala infusion of a CCKB antagonist partially reverses the blockade of extinction seen with CB1 antagonist treatment. Animals were implanted with bilateral cannulae aimed at the BLA 7–10 days before behavioral training. Before each extinction session, all animals were injected with 5 mg/kg SR141716a; in addition to these i.p. injections, either vehicle or 1 μg CR2945 (CR) was bilaterally infused into the BLA (outlined in (a), volume = 0.5 μl over 5 min). Animals receiving intra-BLA CR showed significant extinction retention on post-extinction tests 2 and 3 (b). Animals receiving vehicle did not show significant extinction retention on any of the three post-extinction tests (b). Panel c depicts the change in FPS seen pre- *vs* post-extinction for each of the three tests. Animals receiving intra-BLA CR showed significantly greater reductions in FPS in tests 2 and 3 ($n = 8$ per group; values shown are averages of the first five trials in each test; error bars indicate ± SEM; * denotes $p < 0.05$ comparing pre-extinction FPS to post-extinction FPS within each group).

extinction was observed on days 2 and 3 of testing. Animals that received intra-BLA infusions of CR2945 showed significant extinction retention on the second and third post-extinction tests as compared to their first test and pre-extinction test values (significant main effect of testing day, repeated measures ANOVA $F_{(3, 18)} = 4.344$, $p < 0.05$; *post hoc* tests comparing days 2 and 3 to pre-extinction test and first test, $p < 0.05$; Figure 4b and c). In contrast, vehicle-infused controls failed to show significant extinction on any of the three testing days, suggesting that SR141716A was able to attenuate extinction (repeated measures ANOVA $F_{(3, 18)} = 0.383$, $p = 0.766$). Notably, as the amelioration of the CB1 antagonist effect was slightly less pronounced with intra-amygdala CR2945 infusions compared to systemic administrations, sites other than the amygdala may also be important mediators of the CCK–CB1 interaction. Nonetheless, these data suggest that intra-amygdala blockade of CCK2 receptors is sufficient to overcome the extinction deficit caused by systemic CB1 blockade.

Striatal Infusions of CCK2 Antagonist do not Reverse the CB1 Antagonist-Induced Blockade of Extinction

To confirm that the effect of CR2945 on the reversal of the SR141716A-induced blockade of extinction was due to the amygdala and not another nearby subcortical site, a replication study was performed in exactly the same manner as the above experiment. In this study, cannulae were placed either in the BLA (as above; Figure 5b) or within the striatum as an intracerebral control injection site. Animals were trained and tested as in the preceding experiment, with three groups of animals: (1) those receiving vehicle

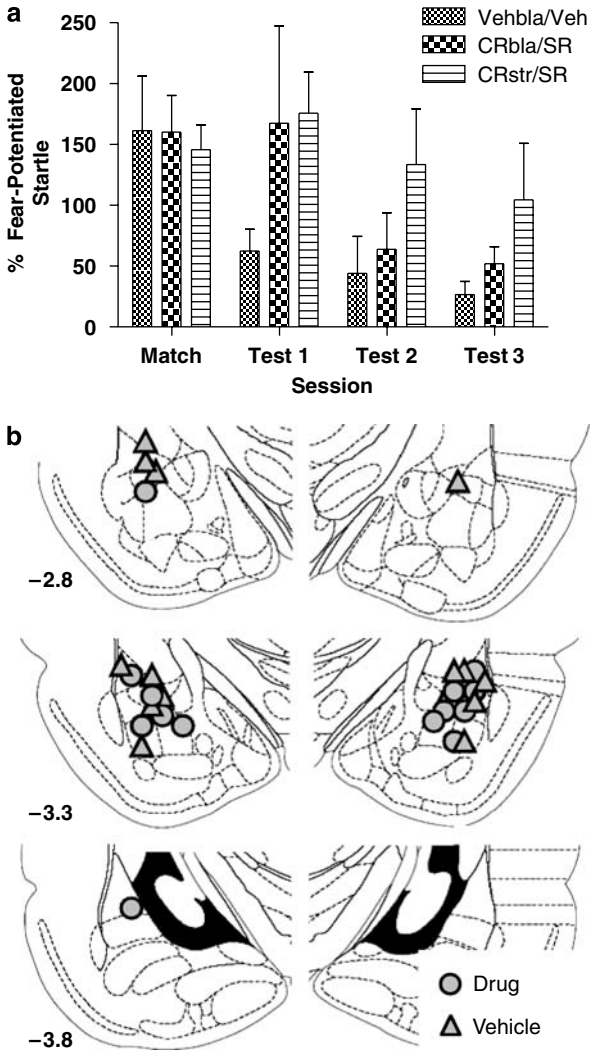

Figure 5 Reversal of systemic cannabinoid antagonist blockade of extinction by local infusion of a CCK antagonist into the BLA but not the striatum. (a) Similar to Figure 4a, animals were implanted with bilateral cannulae aimed at the BLA or striatum (str) 7–10 days before behavioral training. Before each extinction session, all animals were injected with 5 mg/kg SR141716a systemically; in addition to these i.p. injections, either vehicle or 1 μg CR2945 (CR) was bilaterally infused into the BLA or striatum (volume = 0.5 μl over 5 min) before each extinction training session and then tested 48 h later off-drug. Animals given SR141716a systemically and CR2945 into BLA, but not striatum, demonstrate extinction levels similar to those of vehicle-treated animals by the second and third tests. (b) Cannula locations within the BLA (striatal cannula not shown) of rats included within this experiment.

systemically and vehicle intra-BLA (Vehbla/Veh group); (2) those receiving SR141716A systemically and CR2945 intra-BLA (CRbla/SR group); and (3) those receiving SR141716A systemically and CR2945 intrastriatum (CRstr/SR group). We found that the vehicle–vehicle group extinguished rapidly, as expected (Figure 5a; significant main effect of testing day, overall repeated measures ANOVA $F_{(3,87)} = 5.902$, $p < 0.01$; *post hoc* tests comparing day 1 to pre-extinction, $p < 0.05$, days 2 and 3 to pre-extinction, $p < 0.001$). We also found that, as in the reversal experiment above, animals receiving CR2945 in the amygdala (CRbla/SR group) extinguished significantly faster than animals receiving CR2945 infusions in the striatum (CRstr/SR group), which did not show significant extinction during the testing (Figure 5a, *post hoc* tests comparing the intra-amygdala group days 2 and 3 to pre-extinction, $p < 0.05$). This experiment confirms and replicates the finding that CCK2 receptor blockade within the amygdala is sufficient to reverse the systemic effects of CB1 blockade on extinction learning. Furthermore, CCK2 blockade at an extra-amygdalar site, the striatum, does not reverse the effect on extinction of systemic CB1 blockade.

Systemic CCK2 Antagonist Reverses CB1 Antagonist-Induced Deficits in Within-Session Extinction

Previous studies by Marsicano *et al* (2002) have demonstrated that extinction training induces the production of anandamide and 2-AG within the BLA and suggest that activation of CB1Rs during extinction training itself is required for the formation of stable extinction memories. Consistent with this, pre- but not post-training administrations of SR141716A inhibit long-term extinction (Marsicano *et al*, 2002; Suzuki *et al*, 2004; Chhatwal *et al*, 2005). Additionally, the behavior of CB1-knockout mice suggests that they may be impaired in achieving reductions in fear responses during extinction training (Marsicano *et al*, 2002), in turn suggesting that SR141716A may be blocking extinction retention by impairing the dynamic reduction in fear with increasing numbers of non-reinforced CS-alone extinction trials (ie within-session extinction).

As the aforementioned experiments suggested that CR2945 may reverse the effects of SR141716A on extinction retention, we next examined if SR141716A-treated animals would exhibit deficits in within-session extinction and if this impairment would be improved with CR2945 co-administration. In these experiments, rather than presenting 90 lights without shocks during extinction, we presented fear-conditioned animals with 30 light–startle trials intermixed with 30 startle-alone trials. This alteration in our extinction training paradigm allowed us to measure within-session extinction. Thirty minutes before this extinction training/testing session, animals were administered vehicle (100% DMSO), SR141716A (5 mg/kg), or a combination of SR141716A and CR2945 (5 and 3 mg/kg, respectively). To compare the rates of within-session extinction, each animal's FPS was normalized to 100% according to their behavior on the first block of light–startle trials, allowing for comparison across treatment groups and compensating for variations in fear levels at the outset of extinction.

All three groups showed within-session extinction (Figure 6; within-session extinction: overall ANOVA $F_{(9,405)} = 9.891$, $p < 0.001$; vehicle group $F_{(9,153)} = 6.079$, $p < 0.001$; SR141716A-alone group $F_{(9,153)} = 2.594$, $p < 0.01$; SR141716A + CR group $F_{(9,99)} = 7.365$, $p < 0.001$). However, significantly slower extinction was observed in SR141716A-treated animals than in vehicle-treated controls (Figure 6b), an effect that was especially prominent in the middle blocks of the extinction training/testing session. This observation agrees with previous studies (Marsicano *et al*, 2002; Suzuki *et al*, 2004) and provides further evidence that CB1 antagonism impedes within-session extinction.

In contrast, animals co-administered CR2945 and SR141716A showed rates of within-session extinction

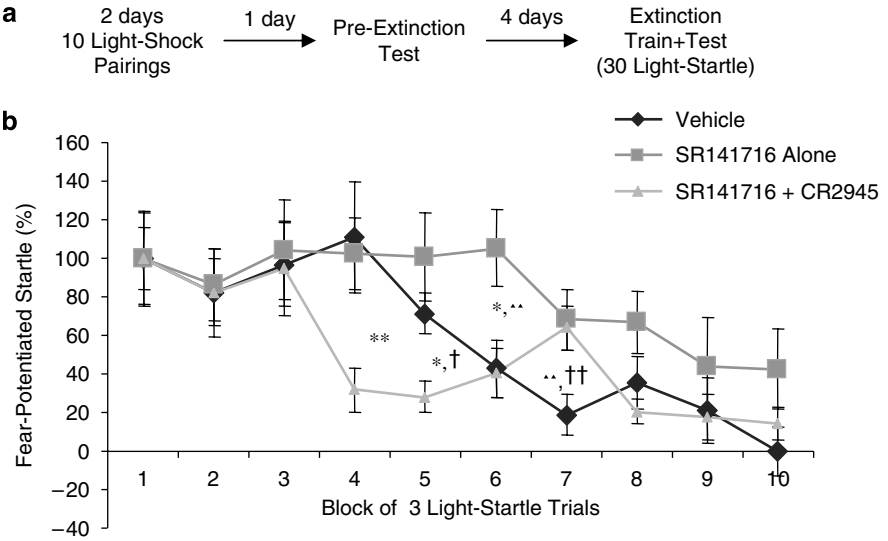

Figure 6 The CB1 antagonist SR141716 impairs within-session extinction in a manner reversible by the CCKB antagonist CR2945. Animals were trained similarly to previous experiments (a), but in lieu of explicit extinction training (light-alone trials), a long (30 light–startle) test was used to determine the rates of within-session extinction in animals receiving vehicle, SR141716a alone (5 mg/kg), or SR141716a and CR2945 (SR141716a + CR; 5 and 3 mg/kg, respectively). FPS in all groups was normalized such that the FPS response during the first block of three trials represented 100% for that group, controlling for differences in starting levels of FPS. SR141716a-treated animals showed less within-session extinction than animals receiving vehicle or SR141716a + CR (b), an effect that was particularly evident in blocks 5–8. In some blocks, SR141716a + CR animals showed significantly different FPS compared to vehicle-treated controls ($n = 18$ each for vehicle and SR141716a-alone groups, $n = 10$ for SR141716a + CR group; error bars indicate ± SEM; in comparisons of SR141716a + CR to SR141716a alone, * denotes $p < 0.05$, ** denotes $p < 0.01$; in comparisons of vehicle to SR141716a alone, ▲▲ denotes $p < 0.01$; in comparison of vehicle to SR141716a + CR, † denotes $p < 0.05$, †† denotes $p < 0.01$).

similar to (and, in some blocks, better than) those of vehicle-treated controls, suggesting that CR2945 may reverse SR141716A-induced deficits in within-session extinction (significant drug × time interaction $F_{(18,405)} = 1.633$, $p < 0.05$; several *post hoc* differences noted in Figure 6b). Additionally, this result suggests that CCK2 antagonism may enhance the rates of within-session extinction.

As the assessment of CCK and CB1 modulation of within-session extinction in these experiments necessitated the testing of animals 'on-drug,' we performed a series of control experiments in which we examined whether the doses of SR141716A and CR2945 used in these studies could affect baseline startle (a measure of basal anxiety) as well as reactivity to footshock, a measure of nociception and general reactivity. Using animals that had been matched for equivalent levels of baseline startle and unconditioned fear to the light, we injected animals with vehicle, SR141716A alone (5 mg/kg), CR2945 (3 mg/kg), or a combination of SR141716A and CR2945 (5 and 3 mg/kg, respectively) 30 min before a test session in which baseline startle and shock reactivity were assessed. We observed that both baseline startle and shock reactivity were similar across all groups (Figure 7), suggesting that the effects of these drugs on within-session extinction proceed without gross alterations of nociception or baseline anxiety.

CCK2 Antagonist Co-administration Reverses CB1 Antagonist-Induced Blockade of Akt Activation Following Extinction

Extinction is an active learning process that is thought to involve many of the same intracellular signaling molecules as fear acquisition. It has previously been shown that extinction training leads to increases in phosphorylated (active) Akt in the amygdala and that the extinction-induced activation of Akt was enhanced when extinction learning was pharmacologically facilitated (Yang and Lu, 2005).

We examined whether SR141716A would prevent the normal induction of phosphorylation of Akt following extinction and whether co-administration of CR2945 with SR141716A would reverse these effects. Rats were fear-conditioned and matched into groups demonstrating similar levels of FPS before extinction (Figure 8a). Thirty minutes before extinction (90 lights without shocks), animals were administered either SR141716A (5 mg/kg) or a combination of SR141716A and CR2945 (5 and 3 mg/kg, respectively). Two control groups were employed: one group received vehicle plus exposure to the training context for the same period of time as extinction-trained controls (context) and the other group received vehicle in addition to extinction training (vehicle group). These rats were then killed 2 h following extinction training and the BLA was rapidly dissected out over ice, homogenized, and stored at −80°C until western blot assays were performed. The 2 h post-extinction time point used here was chosen based on preliminary studies showing that changes in the phosphorylation states of Akt were observable 2 h following extinction training. An overall significant effect of drug treatment and extinction training was seen (ANOVA $F_{(4,51)} = 3.529$, $p = 0.013$).

Levels of phosphorylated Akt appeared similar between SR141716A-treated animals and non-extinguished controls (SR compared to context, $p = 0.44$), suggesting that SR141716A antagonized the extinction-induced activation

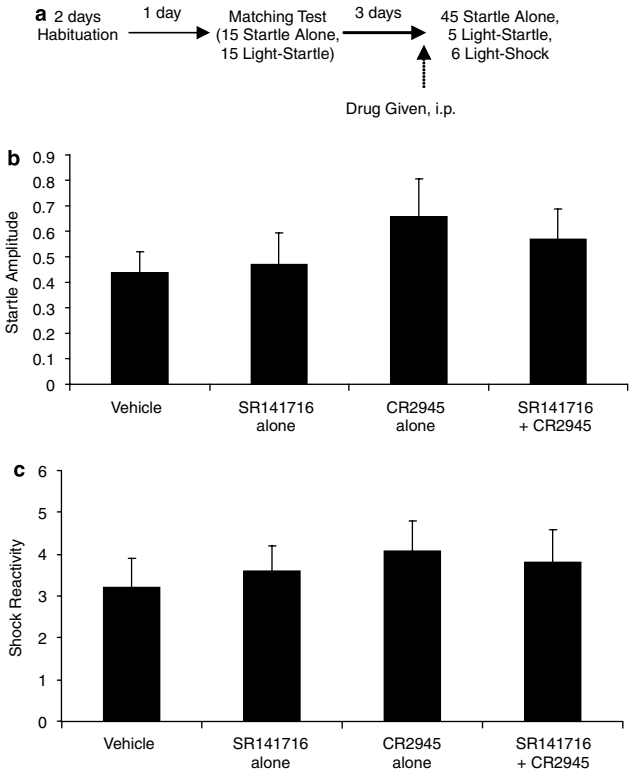

Figure 7 Pre-testing administration of *SR141716a* and/or *CR2945* does not affect baseline startle or shock reactivity. Animals were handled and placed in the training/testing chambers for 2 days for habituation purposes. (a) Following habituation, animals were tested and matched into groups showing equivalent levels of baseline startle and unconditioned fear to the light-CS. Three days later, animals were injected with vehicle (100% DMSO), SR141716a (5 mg/kg), CR2945 (CR, 3 mg/kg), or a combination of SR141716a and CR2945 (Rim + CR, 5 and 3 mg/kg, respectively). Thirty minutes following injection, animals were presented with 30 startle-alone trials, followed by an intermixed session of 5 light–startle compounds and 15 startle-alone trials. At the end of this session, six light–shock compounds were administered to assess shock reactivity (ie accelerometer displacement in response to shock). All four groups showed similar levels of baseline startle (b) and similar levels of shock reactivity (c).

of Akt within the amygdala. However, animals co-administered CR2945 with SR141716A showed significantly higher levels of p-Akt following extinction than animals receiving SR141716A alone ($p = 0.044$), whereas no significant differences were seen between the vehicle + extinction and the group co-administered SR + CR before extinction ($p = 0.52$). This suggests that co-administration of CR2945 may allow extinction-induced increases in p-Akt, even in the presence of SR141716A treatment (Figure 8b and c), a biochemical measure that appears to parallel the behavioral effects of these drugs.

DISCUSSION

We present several lines of evidence to suggest that interactions between the eCB and CCK neurotransmitter systems may play an important role in extinction and that CB1-mediated modulation of CCK2 receptor activation may be a critical component in the CB1 dependency of extinction learning. Specifically, these experiments demonstrate that (1) CCK mRNA and CB1 mRNA are both expressed at a higher level within the BLA, whereas they are relatively absent in the CeA and MeA, (2) i.c.v. infusions of the CCK2 agonist pentagastrin dose-dependently impair extinction; (3) pre-extinction administration of the CB1 antagonist SR141716A potently impairs extinction, and this effect is ameliorated with systemic co-administration of the CCK2 antagonist CR2945, (4) local infusion of CR2945 into the amygdala partially reverses the blockade of extinction seen with systemic SR141716A treatment, (5) increased phosphorylation of Akt in the amygdala is observable 2 h post-extinction training, and this effect is reversed in animals pretreated with SR141716A before extinction; (6) co-administration of CR2945 with SR141716A before extinction training leads to levels of Akt phosphorylation that are similar to control animals; and (7) SR141716A-treated animals showed slower rates of within-session extinction than vehicle-treated controls and animals co-administered SR141716A and CR2945.

In terms of understanding the neural circuitry underlying the process of extinction learning, the present study in combination with the aforementioned anatomical studies describing high concentrations of CB1Rs on the presynaptic terminals of CCK+ interneurons (Marsicano and Lutz, 1999; McDonald and Mascagni, 2001) suggests that the putative synapse of CCK/CB1+ neurons onto BLA pyramidal neurons may be an important locus of plasticity underlying extinction. It should be noted, however, that CB1 modulation of both GABAergic and glutamatergic neurotransmission in the BLA has been demonstrated, suggesting that the interaction between the CCKergic and eCB systems could involve a broad range of synapses (Azad *et al*, 2004). This raises the intriguing possibility that although many pyramidal BLA neurons express low levels of CB1 mRNA, CB1-mediated modulation of glutamate transmission likely plays an important role in extinction.

We have now found in the several experiments described in this paper that the CCK antagonist CR2945 reverses the inhibition of extinction of the CB1 antagonist SR141716A, both when given systemically and via cannula directly into the amygdala. Interestingly, however, CR2945 does not appear to significantly enhance extinction of fear when given alone. There are several possible explanations for this: first, it is possible that the level of extinction that we normally see in these studies is already 'at ceiling,' meaning that we cannot detect further facilitation of extinction or that CR2945 would enhance extinction in animals with low levels of extinction-induced CB1 activation (such as may be the case in chronic stress or in animals with chronic exposure to CB1 agonists). As is the case in stress-related neural systems, there are some pathways that are not able to be modulated at baseline, but which respond differently in stress or anxiogenic situations. With this hypothesis, it is possible that blocking CB1 with systemic antagonist treatment mimics a naturally diminished CB1-mediated process. Similarly, in some studies, D-cycloserine (DCS), an NMDA partial agonist that enhances extinction, has been shown to be more efficacious in states of anxiety than at baseline (Bertotto *et al*, 2006). Additionally, as mentioned earlier, it is likely that CB1-mediated modulation of glutamatergic transmission is also likely to play an important role in extinction.

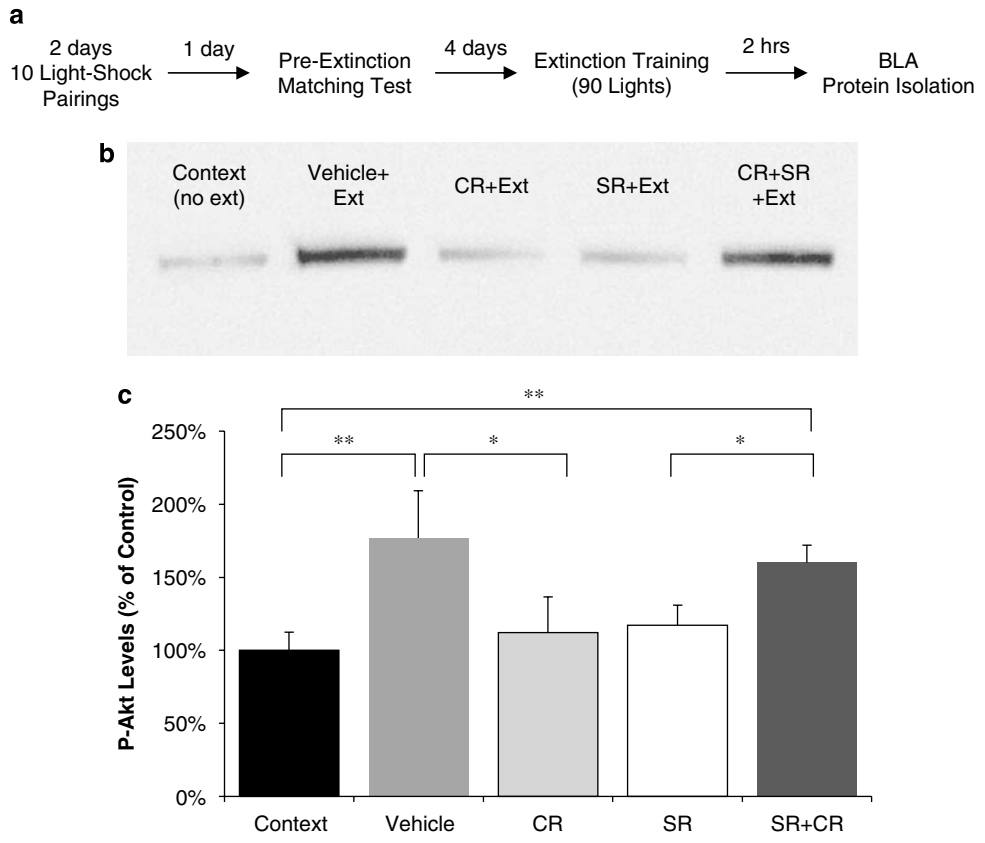

Figure 8 Animals co-administered SR141716A and CR2945 demonstrate higher levels of p-Akt within amygdala following extinction than animals receiving SR141716A alone. (a) Temporal organization of the experiment. (b) Qualitative representation of western blot data from BLA following extinction. (c) A significant increase is found in phospho-Akt in extinction-trained animals, as compared to those receiving context exposure alone. Animals co-administered the CCKB antagonist CR2945 (3 mg/kg) with CB1 antagonist SR141716 (5 mg/kg) before extinction training showed greater p-Akt in the BLA following extinction, as compared to animals who received SR + extinction (SR alone) and animals who were fear-conditioned but not extinction-trained (context) (error bars indicate ± SEM; * denotes $p<0.05$, ** denotes $p<0.01$).

Furthermore, results from both human and animal literature suggest that although CCK antagonists reverse the anxiogenic effects of CCK activation, they do not consistently show such effects when administered alone (Harro, 2006). This is evident in clinical trials of CCK2 antagonists wherein no effect was found following the administration of several CCK2 antagonists in patients with generalized anxiety disorder or panic disorder (Adams *et al*, 1995; Kramer *et al*, 1995; van Megen *et al*, 1997; Pande *et al*, 1999). Several results from the animal literature also support the notion that CCK2 antagonists are not necessarily anxiolytic when administered alone (Dawson *et al*, 1995; Johnson and Rodgers, 1996). These results suggest that the CCK antagonist is not expected to facilitate extinction alone, and are consistent with our hypothesis that perturbation of the endogenous system with the CB1 antagonist is necessary to reveal the underlying behavioral effects of CCK on the extinction of conditioned fear. Further studies aimed at examining pre- and post-synaptic effects of CB1 and CCK2 manipulation on extinction learning hope to further dissect these interacting mechanisms.

The observation that pentagastrin impairs extinction seems to fit well with previous data indicating that CCK receptor agonist treatment is acutely anxiogenic, and with data showing that pentagastrin enhances conditioned fear responses, as measured by FPS (Frankland *et al*, 1996). In the context of the present study, the enhancement of fear expression seen by Frankland *et al* (1996) is particularly interesting in that it suggests that pentagastrin-treated animals may have impairments in adequately reducing their fear responses during extinction training (ie within-session extinction)—a phenotype similar to that seen in CB1 knockouts and in animals administered CB1 antagonists (Marsicano *et al*, 2002; Suzuki *et al*, 2004; Chhatwal *et al*, 2005).

Such a connection may have important clinical implications, as pentagastrin has been shown to be anxiogenic and/or panicogenic in humans, and several modulators of the CB1 and CCK2 receptors are being considered for clinical use. As CCK and CB1 modulation of extinction seems to be a within-session effect (ie an acquisition of extinction effect), it is possible that CCK2 antagonists and/or eCB reuptake inhibitors could be given acutely before exposure-based psychotherapy. Such treatment may be particularly effective in patients manifesting deficits in within-session extinction (or who are especially avoidant in exposure-based sessions) as opposed to extinction retention. Indeed, our group and others are currently investigating the intriguing possibility that genetic differences in the eCB and/or CCKergic neurotransmitter systems may lead to altered susceptibility to trauma-induced psychopathology in humans. Additionally, there is potential for modulators of

eCB or CCKergic transmission to be given in combination with other drugs, such as DCS, which appear to enhance the consolidation of extinction (Walker *et al*, 2002; Ledgerwood *et al*, 2003; Ressler *et al*, 2004). Notably, recent evidence strongly suggests that the acquisition and consolidation of extinction are separable processes (Quirk *et al*, 2000; Santini *et al*, 2001; Chhatwal *et al*, 2006), suggesting that pharmacologic enhancement of either process or both processes may be possible. Although more work is needed to understand the sites at which centrally active CCK2 antagonists can decrease unconditioned and conditioned fear responses, the results of this study suggest that a more complete understanding of CCK2-mediated affective responses may be very useful in the development of anxiolytics that promote extinction learning.

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DISCLOSURE/CONFLICTS OF INTEREST

KJR, MD, and JPC have filed a provisional patent application for the use of enhancers of eCB transmission to augment extinction in clinical settings. Notably, no drugs of this type were used in these studies. KJR has received research support from Lundbeck Pharmaceuticals, LLC. KJR has received consultation fees from Tikvah Pharmaceuticals, LLC.

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Enhancing Cannabinoid Neurotransmission Augments the Extinction of Conditioned Fear

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The endogenous cannabinoid (eCB) system represents a major therapeutic target for the treatment of a variety of anxiety-related disorders. A recent study has demonstrated that pharmacologic or genetic disruption of CB1-receptor-mediated neurotransmission decreases the extinction of conditioned fear in mice. Here, we examined whether CB1 blockade would similarly disrupt extinction in rats, using fear-potentiated startle as a measure of conditioned fear. We also examined whether pharmacologic enhancement of CB1 activation would lead to enhancements in extinction. Our results indicate that systemic administration of the CB1 antagonist rimonabant (SR141716A) prior to extinction training led to significant, dose-dependent decreases in extinction. While the administration of the CB1 agonist WIN 55,212-2 did not appear to affect extinction, administration of AM404, an inhibitor of eCB breakdown and reuptake, led to dose-dependent enhancements in extinction. In addition to showing decreased fear 1 and 24 h after extinction training, AM404-treated animals showed decreased shock-induced reinstatement of fear. Control experiments demonstrated that the effects of AM404 could not be attributed to alterations in the expression of conditioned fear, locomotion, shock reactivity, or baseline startle, as these parameters seemed unchanged by AM404. Furthermore, coadministration of rimonabant with AM404 blocked this enhancement of extinction, suggesting that AM404 was acting to increase CB1 receptor activation during extinction training. These results demonstrate that the eCB system can be modulated to enhance emotional learning, and suggest that eCB modulators may be therapeutically useful as adjuncts for exposure-based psychotherapies such as those used to treat Post-Traumatic Stress Disorder and other anxiety disorders.
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Keywords: amygdala; fear; extinction; PTSD; cannabinoid; phobia

INTRODUCTION

Manipulation of the endogenous cannabinoid (eCB) system has become a major focus of current research, especially in the search for novel therapeutics to treat many common mental illnesses, including anxiety disorders, depression, and drug addiction (Porter and Felder, 2001; Kathuria *et al*, 2003). Indeed, the potential therapeutic value of cannabinoid modulation is underscored by the dense expression of the CB1 receptor in regions known to be important for anxiety and emotional learning, including the amygdala, hippocampus, and throughout the mesolimbic dopamine reward system (Katona *et al*, 1999, 2000, 2001; Freund *et al*, 2003; van der Stelt and Di Marzo, 2003).

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Recent studies of CB1 knockout mice have demonstrated that the genetic deletion of the CB1 receptor leads to increased anxiety in several well-studied measures (Haller *et al*, 2002, 2004a, b; Martin *et al*, 2002). Furthermore, the elegant studies of Marsicano *et al* (2002) have demonstrated that CB1 knockout mice also show profound deficits in the learned inhibition of fear (heretofore referred to as extinction), while the acquisition of the initial fear response was normal. In the same study, the authors demonstrated that pharmacologic blockade of the CB1 receptor led to a similar deficit in extinction in mice, demonstrating the importance of CB1 receptor activation for extinction in mice.

The critical involvement of cannabinoid-mediated transmission in extinction potentially has important clinical implications, as numerous similarities link the expression of fear and anxiety in humans suffering from phobias, Post-Traumatic Stress Disorder (PTSD), and other anxiety disorders to the expression of classically conditioned fear in animals. Perhaps the most important of these similarities is the persistence of fear memories in both humans and animal models. In this context, studying extinction in animals may further the development of experimental

therapeutics for the treatment of these disorders. Indeed, recent results from our laboratory have shown that the administration of a partial NMDA agonist both enhances the extinction of conditioned fear in rodents (Walker *et al*, 2002), and can increase the efficacy of behavioral exposure therapy in human phobics (Ressler *et al*, 2004).

Furthermore, several recent studies have suggested that prolonging the action of released cannabinoids through the inhibition of the enzyme fatty-acid amide hydrolase (FAAH), which is critically involved in cannabinoid catabolism and reuptake, leads to anxiolysis in rodents (Kathuria *et al*, 2003). The results of these and other studies have highlighted the sizable potential of the CB1 receptor and the eCB-degradative enzyme FAAH as targets of experimental therapeutics (eg Porter and Felder, 2001; Kathuria *et al*, 2003).

Given the mounting clinical interest in modulators of the eCB system, we examined whether the CB1 antagonist rimonabant (SR141716A) would block extinction of fear in rats as measured with fear-potentiated startle. We then examined if administration of an agonist (WIN 55,212-2) or of an inhibitor of eCB reuptake and breakdown (AM404) would enhance the extinction of conditioned fear. In so doing, we addressed whether manipulation of the eCB system could lead to enhancements as well as decrements in extinction, a clinically relevant form of fear modulation important in the understanding of emotional learning and in the treatment of anxiety-related behaviors.

MATERIALS AND METHODS

Animals

The procedures used were approved by the Institutional Animal Care and Use Committee of Emory University and in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. A total of 216 adult male Sprague–Dawley rats (Charles River, Raleigh, NC) weighing between 350 and 450 g were used. Animals were housed in pairs in a temperature-controlled (24°C) animal colony, with *ad libitum* access to food and water. They were maintained on a 12 h light/dark cycle with lights on at 0800, with all behavioral procedures performed during the rats' light cycle.

In Situ Hybridization

In situ hybridization was performed as previously described (Ressler *et al*, 2002). A cDNA clone containing the coding sequence of the rat cannabinoid receptor type 1 (CB1) (I.M.A.G.E. expressed sequence tag clone, GI Accession #11375084) was linearized after sequence verification. An antisense riboprobe was generated with T3 RNA polymerase. Slide-mounted sections of snap-frozen rodent brain tissue were postfixed, proteinase K digested, blocked, and hybridized overnight at 52°C with ^{35}S-UTP-labeled riboprobes. After a stringent wash protocol, slides were apposed to autoradiography film and hybridization density was qualitatively assessed.

Fear Conditioning

Animals were trained and tested in $8 \times 15 \times 15$ cm Plexiglas and wire-mesh cages, with floors consisting of four 6.0-mm-diameter stainless-steel bars spaced 18 mm apart. Each cage was suspended between compression springs within a steel frame and located within a custom-designed $90 \times 70 \times 70$ cm ventilated sound-attenuating chamber. Background noise (60-dB wide-band) was provided by a General Radio Type 1390-B noise generator (Concord, MA) and delivered through high-frequency speakers (Radio Shack Supertweeter; Tandy, Fort Worth, TX) located 5 cm from the front of each cage. Sound level measurements (sound pressure level) were made with a Bruel & Kjaer (Marlborough, MA) model 2235 sound-level meter (A scale; random input) with the microphone (Type 4176) located 7 cm from the center of the speaker (approximating the distance of the rat's ear from the speaker). Startle responses were evoked by 50-ms, 95-dB white-noise bursts generated by a Macintosh G3 computer soundfile (0–22 kHz), amplified by a Radio Shack amplifier (100 W; model MPA-200; Tandy), and delivered through the same speakers used to provide background noise. An accelerometer (model U321AO2; PCB Piezotronics, Depew, NY) affixed to the bottom of each cage produced a voltage output proportional to the velocity of cage movement. This output was amplified (model 483B21; PCB Piezotronics) and digitized on a scale of 0–2500 U by an InstruNET device (model 100B; GW Instruments, Somerville, MA) interfaced to a Macintosh G3 computer. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 200 ms after onset of the startle-eliciting stimulus. The CS was a 3.7-s light (82 lux) produced by an 8 W fluorescent bulb (100 μs rise time) located 10 cm behind each cage. Luminosity was measured using a VWR light meter (Atlanta, GA). The US was a 0.5-s shock, delivered to the floorbars and produced by a shock generator (SGS-004; Lehigh Valley, Beltsville, MD). Shock intensities (measured as in Cassella and Davis, 1986) were 0.4 mA. The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads Inc., Newton, CT). Animals were pre-exposed to the chambers for 10 min on each of 2 days prior to training to habituate them to handling and the test chambers and to minimize the effects of contextual conditioning. On 2 consecutive days following habituation, rats were returned to the same chambers and presented with 10 pairings of a light (3.7 s) coterminating with a 0.4-mA, 0.5-s shock (3.6-min intertrial interval).

Matching

At 24 h following the last fear-conditioning session, animals were returned to the same chambers and presented with startle stimuli (50-ms, 95-dB white-noise bursts) in the presence or absence of the light-conditioned stimulus (15 light-startle compounds and 15 startle alone). Increased startle in the presence of the light-CS was taken as a measure of conditioned fear, and the magnitude of the fear response was calculated as the percentage by which startle increased when the light-CS was presented in compound with the startle stimulus *vs* when it was omitted (fear-

potentiated startle or FPS). Using these measurements, animals were divided into groups displaying approximately equal levels of FPS prior to drug treatment and extinction training.

Extinction Training

At 5 days following the last fear-conditioning trial, animals were injected intraperitoneally with a test compound or its vehicle in 1 ml/kg volumes and then immediately returned to the same chambers and presented with 30 or 90 presentations of the light-CS in the absence of footshock (3.7-s light, 30-s intertrial interval). At 1 h following this extinction training session, animals were given a short test consisting of startle stimuli in the presence or absence of the light-CS (2–5 light-startle compounds and 2–5 startle alone, values shown are averages of all trials). At 24 h postextinction training, all animals were tested for the presence of fear-potentiated startle (15 light-startle compounds and 15 startle alone). As animals showed a large amount of extinction within the 24-h testing session (within-session extinction), the FPS values shown for all drug studies are the average FPS during the first five light-startle compounds.

Reinstatement

Previously fear-conditioned and extinction-trained animals were returned to the testing chamber 48 h following extinction training and presented with three footshocks in the absence of the light-CS (0.4 mA, 0.5 s shock, 2 min intertrial interval). Immediately following the unpaired shocks, animals were tested for the presence of fear-potentiated startle (15 light-startle compounds, and 15 startle alone).

Shock Reactivity, Startle, and Activity Measures

A separate group of fear-conditioned animals was injected with AM404, placed in the training/testing chambers, and presented with three unpaired shocks and 42 startle stimuli (0.4-mA, 0.5-s shocks, 95-dB noise-burst startle). The same group of animals was returned to the same chambers 3 days later, injected with vehicle, and presented with an identical behavioral test. The values shown are the mean integrated voltages of the accelerometers measured over 200-ms periods beginning at the onset of either the shocks or the startle stimuli. Additionally, a measure of spontaneous motor activity was derived from the mean displacement of the accelerometers in the 2 min prior to delivery of the first shock, while animals were exploring the chambers.

Drugs

Rimonabant (SR141716A, NIMH Drug Supply Program, Bethesda, MD) and WIN 55,212-2 (Biomol, Plymouth Meeting, PA) were dissolved in 100% DMSO. AM404 (Biomol, Plymouth Meeting, PA) was dissolved in 70% DMSO, 30% PBS. In experiments in which both rimonabant and AM404 were used, all drugs were dissolved in 100% DMSO.

Statistics

Comparisons were made across drug-treatment groups at each test (eg 24-h groups were compared across treatment groups) using ANOVA or Student's *t*-test with drug or dose as the independent measure, and using Fischer's LSD test for *post hoc* analysis.

RESULTS

CB1 is Enriched in the Rat Basolateral Amygdala (BLA)

The BLA has been repeatedly implicated in the extinction of conditioned fear in both direct pharmacological inactivation and augmentation studies (Falls *et al*, 1992; Walker *et al*, 2002; Davis *et al*, 2003). *In situ* hybridization was used to determine if CB1 mRNA was expressed within the rat amygdala and whether it was differentially expressed in the basolateral, medial, and central amygdaloid nuclei. Representative sections from these *in situ* hybridization studies (Figure 1), suggest that CB1 mRNA is highly enriched in the BLA, with very little CB1 mRNA expression seen in the central (CeA) or medial nuclei (MeA) of the amygdala. Additionally, the presence of the mRNA for the CB1 protein within the BLA itself suggests that the CB1-mediated signaling taking place in the BLA is part of the intrinsic neurocircuitry of the BLA. These hybridization results are in close agreement with previous studies using immunohistochemical and hybridization techniques (Katona *et al*, 1999; Marsicano and Lutz, 1999; McDonald and Mascagni, 2001).

CB1 Antagonist Blocks Extinction

The next experiment examined whether pharmacologic antagonism of the CB1 receptor would disrupt extinction in rats and the dose–response relationship for this interaction. Parametric studies were performed to identify a set of behavioral manipulations that could reliably induce extinction in rats. In these studies (outlined in Figure 2a), animals

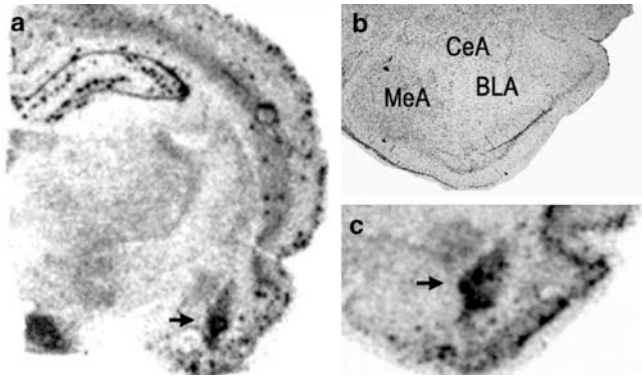

Figure 1 CB1 is densely expressed within rat BLA. Expression patterns of CB1-receptor mRNA are shown following *in situ* hybridization with a ^{35}S-labeled antisense riboprobe. (a) Dense CB1 mRNA expression is seen within amygdala (arrow) and hypothalamus, with more sparse cellular expression throughout hippocampus and cortex. (b) Cresyl violet-stained sections of the temporal lobe. (c) CB1 is most densely expressed within the basolateral amygdala (BLA, arrow). CeA = central amygdaloid nucleus, MeA = medial amygdaloid nucleus.

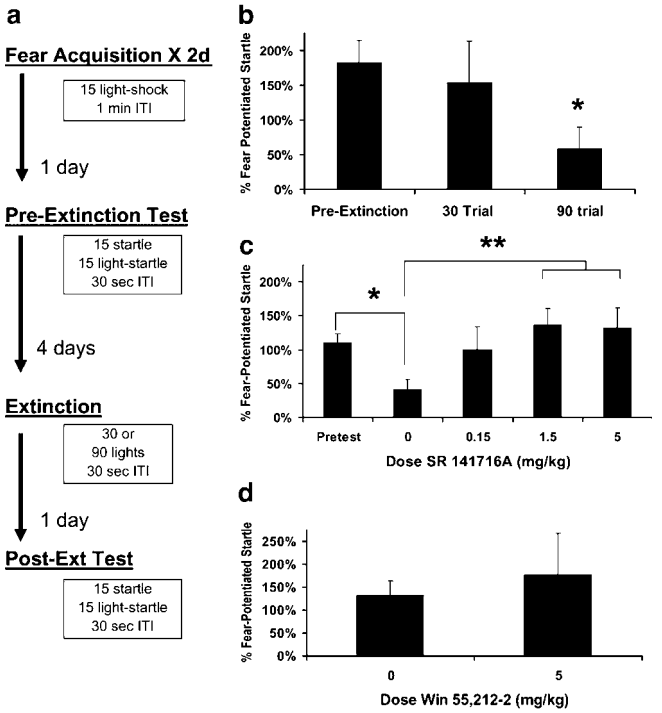

Figure 2 Effect of CB1 antagonist on extinction of fear. (a) Timeline for behavioral experiments. (b) Percent fear-potentiated startle (% FPS) 24 h following extinction training (lights without shocks). Animals receiving 90 light exposures showed significant extinction compared to pretest. (c) % FPS is shown for the pre-extinction test and 24 h postextinction tests of four groups of animals that received SR 141716A (0, 0.15, 1.5, 5 mg/kg, i.p.) prior to extinction training ($n = 16$ for 0, 1.5, and 5 mg/kg groups; $n = 8$ for 0.15 mg/kg group). Only the vehicle group (0 mg/kg) demonstrated significant extinction to the light, and animals receiving the two higher doses of rimonabant displayed significantly greater % FPS than vehicle-treated controls (*denotes $p < 0.05$, **denotes $p < 0.01$). (d) % FPS following extinction in the presence of the CB1 agonist, WIN 55,212-2 ($n = 5$ per group).

showed robust fear conditioning prior to extinction training and varying the number of nonreinforced light-CS presentations decreased the amount of fear animals showed in subsequent testing trials (Figure 2b). We found that 90 trials of nonreinforced lights led to significant extinction retention, whereas only 30 trials led to a more modest, nonsignificant reduction in fear (compared to pre-extinction: 90 trials, $F_{(1,27)} = 4.05$, $p < 0.05$; 30 trials, $p > 0.05$). In all subsequent studies, the 90-trial extinction protocol was used when trying to block extinction, while the weaker 30-trial extinction protocol was used when trying to enhance extinction.

This 90-trial extinction protocol was used to test the effect of systemic administration of the CB1 antagonist rimonabant on extinction in rats. The acute administration of rimonabant to rats immediately prior to extinction training led to a profound disruption of extinction retention, as evidenced by the fact that rimonabant-treated animals showed significantly higher levels of fear in the presence of the light-CS 24 h following extinction training (Figure 2c, ANOVA dose × postextinction FPS, $F_{(3,55)} = 3.40$, $p < 0.05$). This disruption in extinction appeared to be dose-dependent, as animals receiving 1.5 or 5 mg/kg of rimonabant showed significantly higher levels of conditioned fear than vehicle-treated controls, and appeared to show virtually no reduction in conditioned fear following extinction training (*post hoc*, $p < 0.01$ for 1.5 and 5 mg/kg compared to vehicle). The ability of rimonabant to disrupt extinction at the relatively low doses used here suggested that the neural process underlying extinction may be extremely sensitive to the level of CB1 receptor activation during extinction training.

A Direct CB1 Agonist has No Effect on Extinction

The next experiment examined whether the application of the CB1 direct agonist WIN 55-212,2 (WIN) prior to extinction training might enhance extinction retention. A relatively high dose of WIN (5 mg/kg) was administered prior to a 30 trial-extinction training protocol, to determine if increasing CB1 activation would augment the modest extinction normally induced by this weak training protocol. The administration of 5 mg/kg WIN prior to extinction training did not enhance extinction; in contrast, WIN-treated animals actually showed a nonsignificant, but slightly higher, level of conditioned fear 24 h following extinction training (Figure 2d). Notably, the well-documented emergence of prominent locomotor and analgesic effects following administration of higher doses of WIN (eg Tsou *et al*, 1996; Herzberg *et al*, 1997) limited our ability to test the effects of doses of WIN greater than 5 mg/kg.

One explanation for this lack of agonist effect on extinction is that the CB1 receptor could be rapidly downregulated following direct agonist administration (Coutts *et al*, 2001; Hsieh *et al*, 1999). The next experiments examined whether augmentation of endogenously released eCBs, instead of direct agonist administration, would have a different effect.

AM404, an Inhibitor of Cannabinoid Reuptake and Breakdown, Enhances Extinction

In contrast to the potential compensatory decrease in efficacy of CB1-mediated transmission following direct agonist administration, an inhibitor of eCB reuptake or breakdown may enhance extinction by prolonging the action of released eCBs. This, in turn, would lead to increases in activity-dependent CB1-receptor activation.

Consistent with this hypothesis, administration of AM404 prior to 30-trial extinction training led to an enhancement of extinction retention, as AM404 animals showed significantly less fear in the presence of the CS 24 h following extinction training (Figure 3a, main effect of drug treatment $F_{(1,70)} = 4.06$, $p < 0.05$). This enhancement of extinction appeared to be dose-dependent, as animals treated with 10 mg/kg AM404 showed less fear than those treated with 2 mg AM404 and significantly less than vehicle-treated animals (10 mg *vs* control, *post hoc* $p < 0.05$).

A subset of AM404-treated animals was tested 1 h following extinction to assess whether the effects of AM404 were likely taking place during the acquisition phase of extinction. The AM404-induced enhancement of extinction was evident 1 h postextinction, as animals that received the 10-mg/kg dose of AM404 showed significantly less fear than vehicle-treated controls (Figure 3b, ANOVA

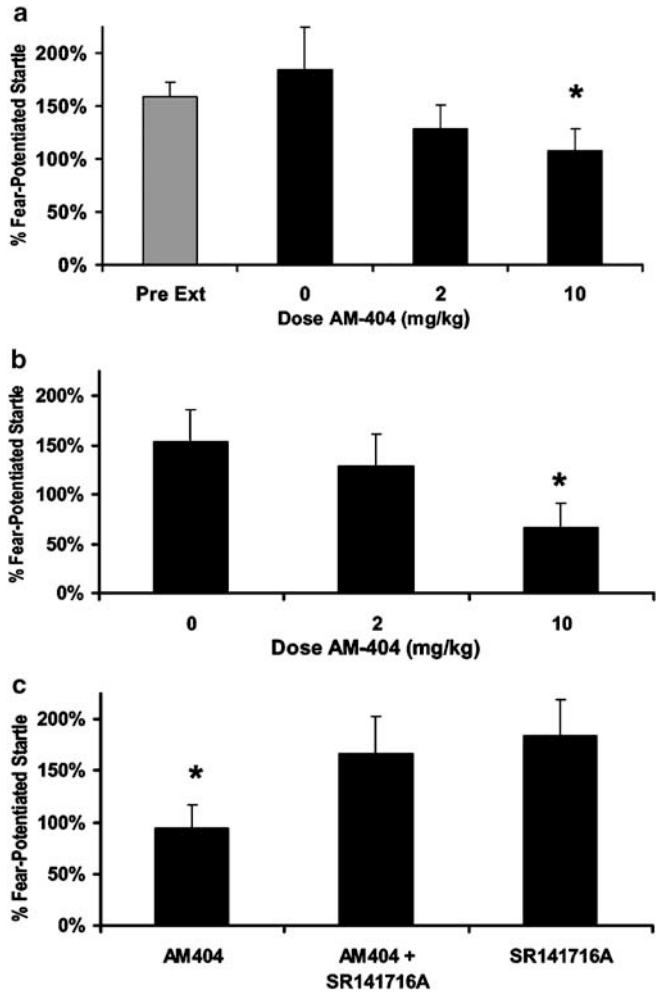

Figure 3 CB1 reuptake inhibitor enhances extinction. AM404 was given prior to extinction training in rats previously fear conditioned as in Figure 2a. (a) % FPS during 24 h postextinction testing in animals that received 0, 2, or 10 mg/kg AM404, i.p., prior to extinction training ($n = 21$ for 0 and 2 mg/kg; $n = 29$ for 10 mg/kg). These data demonstrate increasing extinction (decrement in % FPS) with increasing doses of AM404. (b) % FPS during 1 h postextinction testing in animals that received 0, 2, or 10 mg/kg AM404, i.p., prior to extinction training ($n = 13$ for 0 and 10 mg/kg, $n = 12$ for 2 mg/kg). (c) % FPS during 24 h postextinction testing in animals that received 10 mg/kg AM404 i.p., 5 mg/kg rimonabant i.p., or the combination prior to extinction training ($n = 8$ per group), demonstrating that coadministration of a CB1 antagonist prevented AM404 from enhancing extinction. (*denotes $p < 0.05$)

linear contrast $F_{(1,37)} = 4.89$, $p < 0.05$; *post hoc* comparison, 10 mg/kg *vs* vehicle, $p < 0.05$).

AM404-Dependent Enhancement of Extinction Appears to be Mediated by Activation of CB1 Receptor

AM404 has been implicated in the inhibition of eCB reuptake as well as in inhibiting FAAH (Jarrahian *et al*, 2000; Beltramo *et al*, 2000; Giuffrida *et al*, 2001), but does not itself activate CB1 receptors (eg Beltramo *et al*, 1997). As the enzyme FAAH participates in the breakdown of a number of neuroactive arachidonic-acid derivates (Giuffrida *et al*, 2001) and some have suggested that AM404 may also act at the vanilloid receptor (VR1, Smart and Jerman, 2000), a series of experiments was performed to determine if the AM404-induced enhancement of extinction requires CB1 activation.

Animals were fear conditioned and extinction trained (30 trial extinction) as in the previous study, and prior to extinction training administered 10 mg/kg AM404, 10 mg/kg AM404 + 5 mg/kg rimonabant, or 5 mg/kg rimonabant alone. During testing, animals administered AM404 + rimonabant and rimonabant alone showed virtually no decrease in FPS 24 h following extinction. In contrast, animals treated with 10 mg/kg AM404 alone showed significant extinction ($t_{(26)} = 2.36$, $p < 0.05$ AM404 alone as compared to pre-extinction), and significantly less fear than animals receiving AM404 + rimonabant or rimonabant alone (Figure 3c, $F_{(1,23)} = 5.40$, $p < 0.05$, rimonabant and rimonabant + AM404 groups pooled for comparison). Taken together, these results suggest that the enhancement of extinction seen in AM404-treated animals is mediated via CB1-receptor activation.

AM404-Induced Enhancement of Extinction Requires Cue-Exposure and is not Due to Drug-Induced Changes in the Expression of Conditioned Fear

A series of control experiments was performed to rule out the possibility that AM404 administration itself could lead to decreases in the expression of conditioned fear, even in the absence of cue re-exposure during extinction training. To this end, a parallel set of rats was fear conditioned and matched for equivalent levels of FPS as in the above studies. On the day on which extinction training was to be performed, animals were administered 10 mg/kg AM404, 5 mg/kg rimonabant, or vehicle, but cue re-exposure was omitted. At 1 h following drug administration, animals were tested for FPS using a procedure similar to the above studies. The results from these studies indicate that the highest doses of AM404 and rimonabant used here had no effect on FPS if cue-exposure was omitted, as all drug groups showed similar levels of conditioned fear 1 h following drug administration (Figure 4a).

AM404 Treatment Does not Lead to Obvious Analgesic or Locomotor Effects

To better understand the behavioral effects engendered by AM404 treatment, a series of control experiments was performed. These included testing the effects of 10 mg/kg AM404 on (1) shock reactivity as a measure of pain sensitivity, (2) baseline startle as one measure of anxiety, and (3) general motor activity within the training chambers. Animals were fear conditioned and then returned to the training chamber several days later and administered 10 mg/kg AM404. Following drug administration, animals were presented with three shocks and 42 startle stimuli identical to those used in the above studies. Subsequently, the same animals were returned to the testing chamber 3 days later, injected with vehicle, and similarly tested. The results from these studies (Figures 4b–d) showed that the administration of AM404 had little effect on shock reactivity or overall locomotor activity levels in the testing chamber ($p > 0.5$ for both comparisons). The apparent decrease in baseline startle observed following AM404 administration

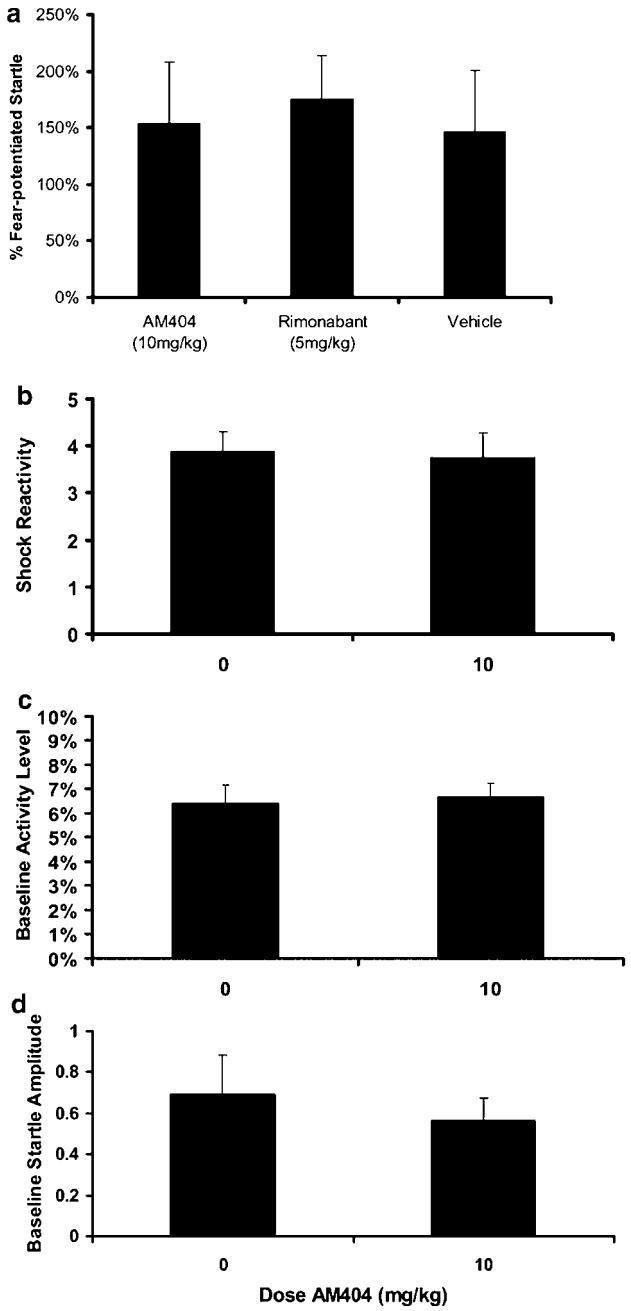

Figure 4 AM404 effect on extinction is independent of effects on the expression of conditioned fear, pain, locomotion, and baseline anxiety. (a) Animals fear-conditioned as in the above studies were administered AM404 (10 mg/kg), rimonabant (SR141716A, 5 mg/kg), or vehicle and 1 h later tested for FPS (cue re-exposure was omitted, $n = 8$ per group). Neither AM404 nor rimonabant treatment led to significant alterations in the expression of conditioned fear when cue re-exposure was omitted. (b) Average shock reactivity is shown in arbitrary units and represents the average response to three footshocks. (c) Average baseline activity level is shown in arbitrary units as determined by mean displacement of accelerometers during the 2 min prior to the delivery of any shocks in the test chambers. (d) Average baseline startle amplitude shown in arbitrary units during the presentations of startle stimuli ($n = 8$ per group for b–d).

was not significant ($p = 0.45$). Taken together, these results suggest that the administration of AM404 at the doses used in this study are insufficient to generate obvious motor or analgesic effects, and do not likely affect anxiety levels as measured by baseline startle amplitude.

AM404 Treatment Prior to Extinction Training Decreases Shock-Induced Reinstatement of Fear

Shock-induced reinstatement was then examined 2 days following treatment with AM404 or vehicle during extinction. Previous studies have shown that the level of fear following reinstatement is dependent both on the level of the stressor and the amount of previous extinction, as long as the stressor is delivered in the same context as the original training context (Rescorla and Heth, 1975; Bouton and King, 1983). Thus, diminished reinstatement following extinction serves as an additional measure of the strength of the extinction process. As animals were matched for equivalent FPS prior to extinction training, the susceptibility of animals to reinstatement can be taken as a secondary measure of the strength of extinction training, and perhaps as a preliminary measure of the resiliency of these inhibitory extinction memories to stressors.

In these studies, animals that had previously been fear conditioned, extinction trained, and tested for extinction retention were returned to the training chambers and presented with three footshocks (in the absence of light-CS presentation) followed by a test for the presence of FPS to the light-CS. During these reinstatement tests, AM404-treated animals showed less reinstatement-induced conditioned fear, whereas control animals showed a transient but robust re-emergence of conditioned fear following the unpaired footshocks. This effect was especially prominent during the first two testing trials, where vehicle-treated animals showed significantly more fear to the light CS than their AM404-treated counterparts (Figure 5a $t_{(63)} = 4.5$, $p < 0.05$, 2 and 10 mg/kg AM404-treated groups pooled for comparison to vehicle). Additionally, examination of within-session extinction demonstrated a significant decrease in FPS among vehicle-treated groups, but little change among AM404-treated groups (Figure 5b, repeated measures ANOVA, Trial × Drug interaction, $F_{(1,62)} = 5.67$, $p < 0.02$). Note that within this period of extinction testing, neither group reached terminal levels of extinction.

DISCUSSION

These experiments demonstrate that: (1) CB1 mRNA is expressed densely and relatively specifically within the rat BLA, a region implicated in the extinction of conditioned fear, and there is little expression seen in the medial and central nuclei; (2) systemic application of a specific CB1 antagonist (SR 141716A) to rats dose-dependently blocks the extinction of fear as it does in mice; (3) this dose-dependent blockade of extinction is robust and easily measured using fear-potentiated startle as a measure of fear; (4) systemic application of AM404, an inhibitor of eCB breakdown and membrane transport, dose-dependently enhances extinction of fear as measured at different times following cue re-exposure; (5) this enhancement of extinction is not likely due to changes in baseline anxiety, locomotion, or nociception; (6) the enhancement of extinction with AM404 is likely CB1-dependent, as this

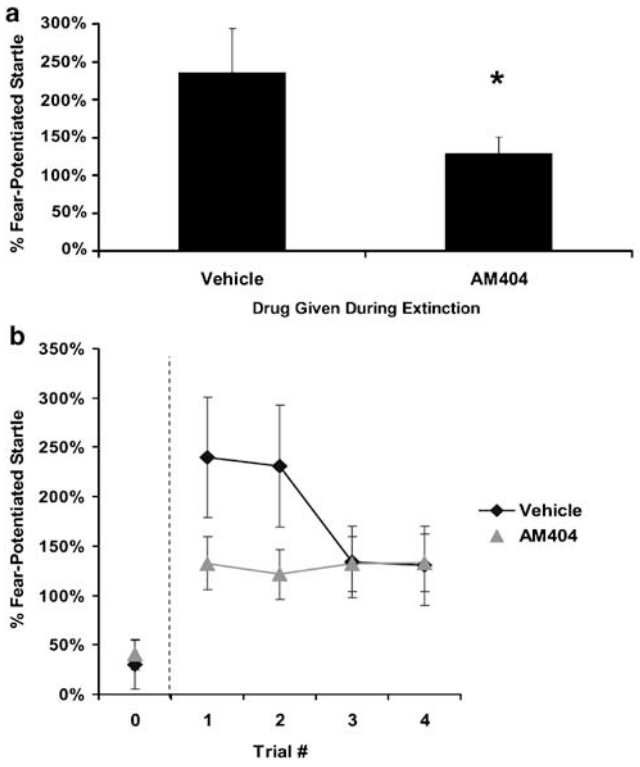

Figure 5 AM404-enhanced extinction decreases shock-induced reinstatement. (a) % FPS is shown during testing following reinstatement with three footshocks. Animals had received vehicle ($n=21$) or AM404 (2-mg and 10-mg groups combined, $n=42$) prior to extinction training (ie, 48 h prior to reinstatement). Animals that received AM404 prior to extinction training demonstrated significantly less % FPS following reinstatement than did control animals. (*denotes $p<0.05$) (b) Within-session extinction is shown for the first four trials during the testing of FPS following the reinstatement experiment described in (a). The terminal level of FPS in the last testing trial prior to reinstatement (shown as trial 0) indicates that vehicle and AM404-treated animals showed similar FPS levels prior to reinstatement.

effect is blocked almost completely by coadministration of rimonabant; and (7) this enhancement of extinction appears to diminish reinstatement of fear following footshock.

As has been shown previously, eCB function is normally required for extinction of fear in mice using both pharmacological (Marsicano *et al*, 2002; Suzuki *et al*, 2004) and genetic approaches in CB1 knockout animals (Marsicano *et al*, 2002). In this study, we demonstrate that blockade of the CB1 receptor with rimonabant also prevents extinction of fear in rats as measured with the fear-potentiated startle paradigm.

That a similar effect did not occur with systemic application of the direct CB1 agonist, WIN 55,212-2, could be due to a rapid downregulation or desensitization of the CB1 receptor following prolonged activation (Coutts *et al*, 2001; Hsieh *et al*, 1999). Direct CB1 agonists have been shown to lead to downregulation of CB1 receptors (Hsieh *et al*, 1999) and to uncoupling of the CB1 receptor from its effector G-protein, G_i (Mato *et al*, 2004). The presence of a physiologic mechanism to rapidly decrease the efficacy of CB1-mediated transmission would have important implications for future and ongoing clinical studies examining the use of direct CB1 agonists for the treatment of anxiety disorders and drug addiction. Future studies examining a broader range of doses of WIN 55,212-2 would be necessary to definitely determine if a lower dose of this drug might enhance extinction without leading to potential CB1 downregulation. These concerns make the use of inhibitors of cannabinoid reuptake and of FAAH more clinically attractive, since the cannabinoid system has very low basal levels of activation (Giang and Cravatt, 1997; Cravatt *et al*, 2001).

Reinstatement of fear is one of the principle behavioral processes upon which the idea is based that extinction does not lead to an 'erasure' of memory, but rather to a parallel inhibitory process that masks the previous fear memory (for a review, see Myers and Davis, 2002). We found that animals that had received AM404 during the extinction exposure showed less initial fear-potentiated startle when tested following reinstatement in the absence of any drug (Figure 5a and b). This finding is consistent with previous findings in which pharmacological enhancement of extinction with D-cycloserine (DCS) leads to less reinstatement (Ledgerwood *et al*, 2004), and provides further support for the hypothesis that the extinction seen following AM404 treatment is more robust and less susceptible to subsequent stress than the extinction seen in vehicle-treated controls. Future studies using more clinically relevant stressors and contexts are needed to clarify whether AM404 reduces susceptibility to reinstatement in a therapeutically useful way.

Drugs that can be given only at the time of extinction may provide for a new and powerful way to treat anxiety disorders. We and others have previously shown that extinction, which is known to be NMDA-dependent (Falls *et al*, 1992; Santini *et al*, 2001; Suzuki *et al*, 2004), can be enhanced with systemic or local administration into the amygdala of DCS, a partial NMDA agonist (Walker *et al*, 2002; Ledgerwood *et al*, 2003, 2004). Follow-up clinical trials have now demonstrated that this approach may be successful in humans as well (Ressler *et al*, 2004; Rothbaum and Davis, 2003).

AM404 and other inhibitors of the anandamide transporter, such as the newly identified AM1172 (Fegley *et al*, 2004), may enhance the process of extinction through an alternate mechanism. Since DCS can potentially activate all NMDA receptors, it is possible that it could enhance fear learning as well as extinction, although this has not been observed experimentally (Ledgerwood *et al*, 2003). In contrast, the CB1-receptor knockout mice have no decrement in fear learning, and pharmacological blockade of CB1 does not affect fear conditioning (Marsicano *et al*, 2002). Therefore, it appears that the activation of the cannabinoid system may be relatively specific to effects on inhibitory learning within the BLA, and it may not be required for or have a substantial impact on excitatory learning such as fear conditioning. This idea fits well with recent physiologic studies suggesting that CB1 activation may lead to enhanced LTD by presynaptically decreasing GABA release within the BLA (Azad *et al*, 2003; Chevaleyre and Castillo, 2003), perhaps via coincident activation of both presynaptic NMDA and cannabinoid receptors, as has been elegantly shown in the neocortex (Sjostrom *et al*, 2003). Lastly, it has been shown that extinction learning critically requires the activation of MAP kinase and calcineurin (Lu *et al*, 2001;

Lin *et al*, 2003a, b), and that CB1-receptor activation regulates the activity of a variety of kinases and phosphatases within the BLA (including MAP kinase and calcineurin, Cannich *et al*, 2004), providing a putative mechanistic link between eCB modulation and the plasticity underlying extinction.

Taken together, the findings in the present study suggest that augmenting eCB-mediated neurotransmission by inhibition of eCB transport or breakdown may provide a novel mechanism for enhancing the extinction of fear. As such, eCB reuptake inhibitors may serve as useful adjuncts in the treatment of anxiety disorders (such as PTSD, panic disorder, and OCD) as well as drug addiction and other disorders that respond to behavioral treatments utilizing extinction processes.

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Effects of intra-amygdala infusion of CB1 receptor agonists on the reconsolidation of fear-potentiated startle

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Research

Effects of intra-amygdala infusion of CB1 receptor agonists on the reconsolidation of fear-potentiated startle

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The cannabinoid CB1 receptor has been shown to be critically involved in the extinction of fear memory. Systemic injection of a CB1 receptor antagonist prior to extinction training blocked extinction. Conversely, administration of the cannabinoid uptake inhibitor AM404 facilitated extinction in a dose-dependent manner. Here we show that bilateral infusion of CB1 receptor agonists into the amygdala after memory reactivation blocked reconsolidation of fear memory measured with fear-potentiated startle. The effect was dose-dependent and could be blocked by AM251, a specific CB1 receptor antagonist. In contrast, the effect of CB1 agonists on reconsolidation was no longer seen if memory reactivation was omitted. Concomitant with block of reconsolidation, CB1 agonist-treated animals did not exhibit shock-induced reinstatement or spontaneous recovery of fear. The absence of recovery was not attributable to permanent damage to the amygdala in WIN-treated rats, nor did the effect result from alteration of baseline startle or shock reactivity. These results suggest that CB1 agonists could impair fear memory via blocking reconsolidation.

Synthetic and endogenous cannabinoids have profound effects on the central neurons. They inhibited pain (Pertwee 2001) and reduced neuronal damage in models of ischemia and traumatic brain injury (Panikashvili et al. 2001). They impaired memory in animals, particularly in hippocampus-dependent tasks such as an eight-arm radial maze, spatial alteration in a T-maze, and delayed matching/non-matching to a position task with lever presentation (Lichtman et al. 1995; Davis et al. 2002). On the other hand, SR141716A, a specific antagonist of the cannabinoid CB1 receptor, blocked the disruptive effects of cannabinoids on rate and accuracy of responding (Brodkin and Moerschbaecher 1997). Cannabinoids produce marked alterations in behavior and mood in animals and humans. Administration of a CB1 antagonist elicited an anxiety-like response (Navarro et al. 1997), whereas active inhibitors of fatty acid amide hydrolase (FAAH), which catalyzes endogenous cannabinoid anadamide hydrolysis, induced anxiolytic effects in rats (Kathuria et al. 2003).

Pavlovian fear conditioning is a behavioral procedure in which a cue (conditioned stimulus, CS) comes to induce a fear response when it is repeatedly paired with a noxious stimulus, often a foot-shock (unconditioned stimulus, US). Fear conditioning is not only a sensitive measure of anticipatory fear or anxiety but is also a leading behavioral paradigm for studying the neural mechanisms through which emotional memory is formed and stored (Davis 2000; LeDoux 2000). Extinction, on the other hand, refers to gradual disappearance of the previously acquired responses if animals are exposed only to the cue without pairing with a shock (Rescorla 2001; Myers and Davis 2002). Recently, endocannabinoids were demonstrated to be critically involved in the extinction of fear memory because mutant mice lacking CB1 receptors were specifically impaired in extinction (Marsicano et al. 2002).

Many observations in animal studies, including spontaneous recovery with time (Bouton and Peck 1989), reinstatement after unpaired US presentations (Rescorla and Heth 1975), and renewal with context change (Bouton and King 1983), indicate that extinction is a new inhibitory learning, which leaves the original memory intact (Quirk et al. 2000; Herry and Garcia 2002; Myers and Davis 2002; Maren and Quirk 2004). It has been shown that treatment of rats with an inhibitor of cannabinoid reuptake, AM404, enhanced extinction (Chhatwal et al. 2005). However, animals that had received AM404 during extinction training exhibited less reinstatement effect. It is possible that extinction seen following AM404 treatment was more robust and less susceptible to subsequent US reinstatement. Alternatively, it may suggest the possibility of additional mechanisms. Following retrieval, memory became labile for a period before being reconsolidated and re-stored. Thus, in theory, memory would not return after a block of reconsolidation (Duvarci and Nader 2004). Extinction training usually consisted of CS-alone trials that induced memory retrieval. Therefore, it is reasonable to speculate that CB1 receptor agonists may act on the reconsolidation of fear memory.

Results

On day 1, rats were conditioned with 10 light–shock pairings. On day 2, they were infused with vehicle or a CB1 receptor agonist, WIN55212–2 (WIN, 1 or 11 μg per side), bilaterally into the amygdala within 1 h after a retention test (Test 1). Memory was assessed 24 h after Test 1 (Test 2). Figure 1A shows that infusion of WIN resulted in an impairment of fear memory. Startle potentiations were 171.4% ± 8.3% ($n = 6$) in vehicle controls, 99.0% ± 13.6% (1 μg per side, $n = 5$), and 46.0% ± 7.7% (11 μg per side, $n = 10$) in WIN-treated animals. The ANOVA for startle scores showed a significant effect for group ($F_{(2,18)} = 48.17$, $P < 0.001$), and post hoc *t*-tests showed that the two WIN groups differed from the vehicle group ($P < 0.001$). Furthermore, less startle reflex occurred in the high-dose group than in the low-dose group ($P < 0.01$), indicating a dose-dependent effect. The infusion cannula tip locations are shown in Figure 1B. Only rats with cannula tips at or within the boundaries of LA and BLA were included in the data analysis.

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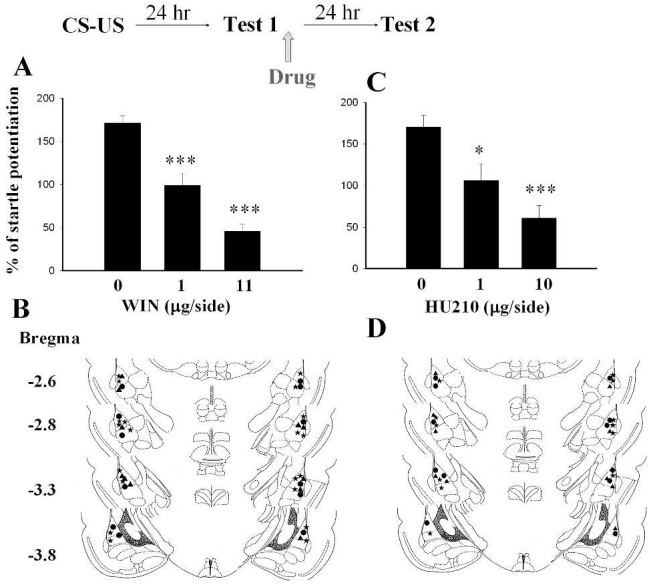

Figure 1. CB1 receptor agonists block reconsolidation of fear memory. (*A*) Rats were infused with vehicle ($n = 6$), 1 µg of WIN ($n = 5$), or 11 µg of WIN ($n = 10$) within 1 h after the test, and memory retention was assessed 24 h later. ***$P < 0.001$ vs. vehicle. (*B*) Cannula tip placements from rats infused with vehicle (●), 1 µg of WIN (▲), or 11 µg of WIN (★) in the experiments shown in *A*. (*C*) Dose–response relationship of HU210 on reconsolidation. *$P < 0.05$, ***$P < 0.001$ vs. vehicle. (*D*) Cannula tip placements from rats infused with vehicle (●), 1 µg of HU210 (▲), or 10 µg of HU210 (★) in the experiments shown in *C*.

A similar result was obtained with another CB1 agonist, HU210. Post-test infusion of HU210 significantly attenuated startle reflex. Startle potentiations were 170.5% ± 14.1% ($n = 6$) in vehicle controls, 106.4% ± 19.9% (1 µg per side, $n = 5$, $P < 0.05$ vs. vehicle), and 61.1% ± 15.2% (10 µg per side, $n = 6$, $P < 0.001$ vs. vehicle) in HU210-treated animals (Fig. 1C). Cannula tip placements are shown in Figure 1D.

AM251 is a selective CB1 antagonist. To ensure that the memory-impairing effect of WIN was mediated by the CB1 receptor, we determined whether AM251 could reverse the effects of WIN and HU210. AM251 (20 µg per side) and WIN (11 µg per side) were sequentially infused into the amygdala with an interval of 20–25 min. As shown in Figure 2, AM251 blocked the effects of WIN and HU210 (10 µg per side) such that there was no difference in the amount of startle amplitude between the vehicle and WIN/AM251 groups ($t_{(12)} = 0.18$, $P = 0.86$) and between the vehicle and HU210/AM251 groups ($t_{(7)} = 0.68$, $P = 0.52$). As a control, vehicle and AM251 also were sequentially infused into the amygdala to investigate the effect of AM251 on reconsolidation. The result showed that there was no difference between the vehicle and veh/AM251 groups ($t_{(8)} = 0.32$, $P = 0.75$), suggesting that AM251 by itself did not affect reconsolidation and that concentrations of endocannabinoids were below threshold during the retention test to activate CB1 receptors.

We repeated the experiments to determine the effects of WIN on post-reactivation of short-term memory (PR-STM) at 4 h and long-term memory (PR-LTM) at 24 h after Test 1. An ANOVA comparing the drug group across trials (PR-STM and PR-LTM) demonstrated a significant interaction ($F_{(3,20)} = 11.94$, $P < 0.001$). Newman-Keuls post hoc analysis revealed that the WIN group was significantly different from the vehicle group both in the PR-STM ($P < 0.05$) and PR-LTM ($P < 0.001$) (Fig. 3). Taken together, these results indicate that CB1 receptor agonists impair fear memory when given shortly after memory reactivation.

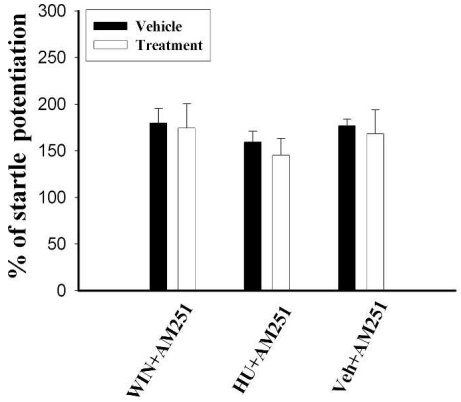

Figure 2. Block of the effect of CB1 agonists on reconsolidation by AM251. AM251 (20 µg per side) was administered 20–25 min before WIN (11 µg per side) or HU210 (10 µg per side). There was no difference in the amount of startle amplitude between the vehicle and WIN/AM251 groups ($t_{(12)} = 0.18$, $P = 0.86$) and between the vehicle and HU210/AM251 groups ($t_{(7)} = 0.68$, $P = 0.52$). AM251 and vehicle were also infused into the amygdala, and there was no difference between the vehicle and veh/AM251 groups ($t_{(8)} = 0.32$, $P = 0.75$).

To determine whether the observed impairment of fear memory required memory reactivation, we omitted Test 1. Conditioned rats were infused with WIN, HU210, or vehicle in the absence of Test 1. Memory retention was assessed 24 h after drug application. Figure 4 shows that neither WIN (11 µg per side) nor HU210 (10 µg per side) had an effect on the startle reflex. Furthermore, WIN still failed to induce extinction even though the dose was increased to 33 µg per side. These results suggest that the effects of WIN and HU210 require memory reactivation as demonstrated by the lack of amnesia when Test 1 is omitted.

To examine the possibility that WIN might damage the amygdala neurons, we performed a histological analysis. Figure 5A shows that there was no evidence of increased gliosis or cell loss in vehicle- or WIN-treated rats. We further determined whether WIN induced cell apoptosis by staining neurons with Hoechst 33,342. WIN or vehicle was infused into the amygdala, and 24 h later apoptotic features including dense chromatin condensation and nuclear pyknosis were examined with a fluorescence microscope. There was no difference in abnormal nuclei-positive cells between vehicle- and WIN-treated animals (Fig. 5B).

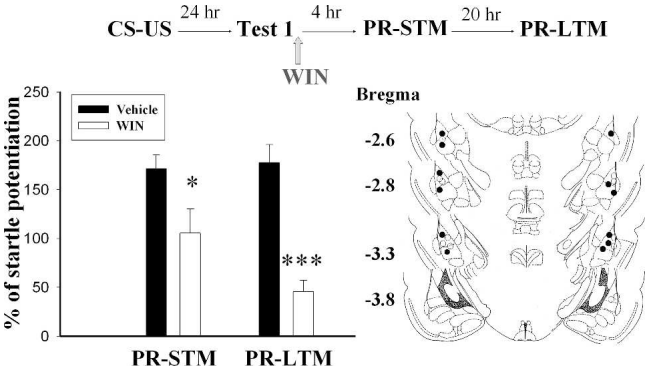

Figure 3. Effects of post–Test 1 infusion of WIN on STM and LTM. Rats were infused with vehicle or 11 µg of WIN within 1 h after the test, and STM was assessed at 4 h and followed by LTM at 24 h after administration of WIN. *$P < 0.05$, ***$P < 0.001$ vs. vehicle. Cannula tip placements from rats infused with vehicle (○) or WIN (●).

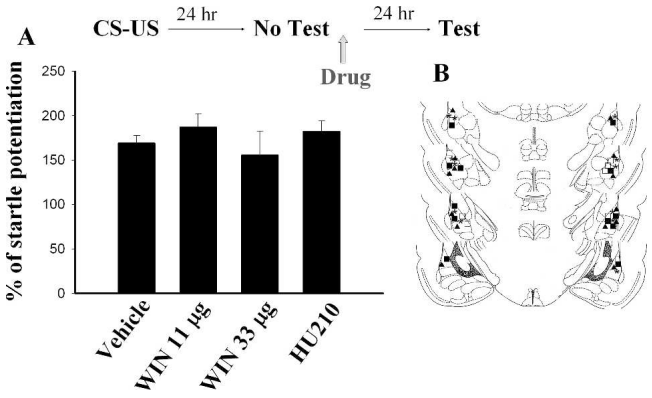

Figure 4. Requirement of memory retrieval for the action of CB1 agonists. (*A*) There was no difference in startle reflex between vehicle- and WIN- or HU210-treated rats when Test 1 was omitted. (*B*) Cannula tip placements from rats infused with vehicle (▲), 11 µg WIN (□), 33 µg WIN (■), or HU210 (★).

We assessed whether WIN-treated rats exhibited reinstatement of fear memory. Rats were trained according to our previous reconsolidation paradigm and then tested for memory recovery by application of a reminder shock (Fig. 6A). Vehicle control rats were divided into two groups with or without exposing to CS-alone trials that led to extinction. An ANOVA on Test 1, PR-LTM, and reinstatement showed a significant interaction with drug treatment ($F_{(5,33)} = 24.12$, $P < 0.0001$). Post hoc comparisons revealed that Test 1 scores were the same for the vehicle and WIN groups ($P > 0.05$). However, WIN rats demonstrated less startle reflex than controls on both PR-LTM ($P < 0.001$) and reinstatement ($P < 0.001$). In contrast, subsequent exposure of vehicle extinction rats to 10 foot-shocks reinstated the startle. Furthermore, there was no increase in the startle amplitude of WIN-treated animals after the reminder shock ($t_{(6)} = 1.21$, $P = 0.27$). To rule out the possibility that the lack of recovery was attributable to WIN-induced damage to the amygdala, five out of seven WIN-treated rats were retrained. Figure 6B shows that startle reflex in all five WIN-treated rats was significantly increased to levels (183.1 ± 13.9, $t_{(4)} = 5.98$, $P < 0.005$ vs. reinstatement) comparable with control animals on Test 1. This result suggests that the lack of reinstatement is not attributable to the inability of animals to learn.

Similar experiments were performed with HU210 (10 µg per side). ANOVA analysis on Test 1, PR-LTM, and reinstatement showed a significant interaction with drug treatment ($F_{(5,27)} = 14.14$, $P < 0.0001$). Post hoc comparisons revealed that Test 1 scores were the same for both groups ($P > 0.05$), whereas the HU210 rats demonstrated less startle reflex than controls on both PR-LTM ($P < 0.001$) and reinstatement ($P < 0.001$). In addition, there was no increase in the startle amplitude of HU210-treated animals after a reminder shock ($t_{(4)} = 0.57$, $P = 0.60$). 5 d later, these HU210-treated rats were retrained and, as shown in Figure 6B, the level of startle potentiation was increased to 151.7% ± 18.3% ($t_{(4)} = 5.50$, $P < 0.01$ vs. reinstatement).

We examined whether the memory would recover spontaneously from reactivation amnesia in WIN-treated rats. Animals were trained according to our previous reconsolidation paradigm and then tested for memory recovery 7 d after training. To match the levels of startle in the WIN group, vehicle control rats were given 30 trials of CS-alone extinction training ~30 min after Test 1. As shown in Figure 7B, testing animals 7 d after training revealed a recovery of startle in vehicle controls. In contrast, the conditioned responses of the WIN (11 µg per side) and HU210 (10 µg per side) groups were significantly less than vehicle controls 7 d after training (WIN: $t_{(10)} = 2.40$, $P < 0.05$; HU210: $t_{(10)} = 2.95$, $P < 0.02$), indicating an inhibition of spontaneous recovery by CB1 agonists.

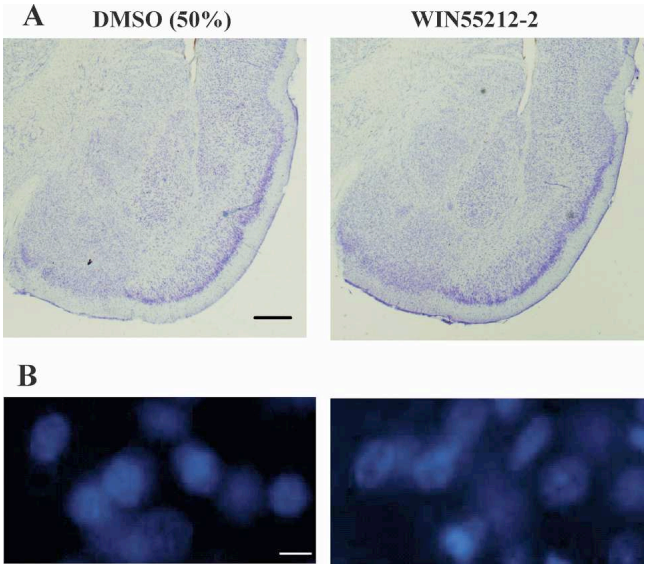

Figure 5. WIN55212–2 did not lesion the amygdala. (*A*) Representative photomicrographs show amygdala slices from rats infused with DMSO (*left*) or WIN (*right*). There was no evidence of increased cell loss or gliosis in the amygdala in the DMSO or WIN-treated animals. Bar, 0.5 mm. (*B*) WIN (11 µg/side) or vehicle were infused into the amygdala, and 24 h later morphological studies were conducted by Hoechst 33,342 staining. Bar, 10 µm.

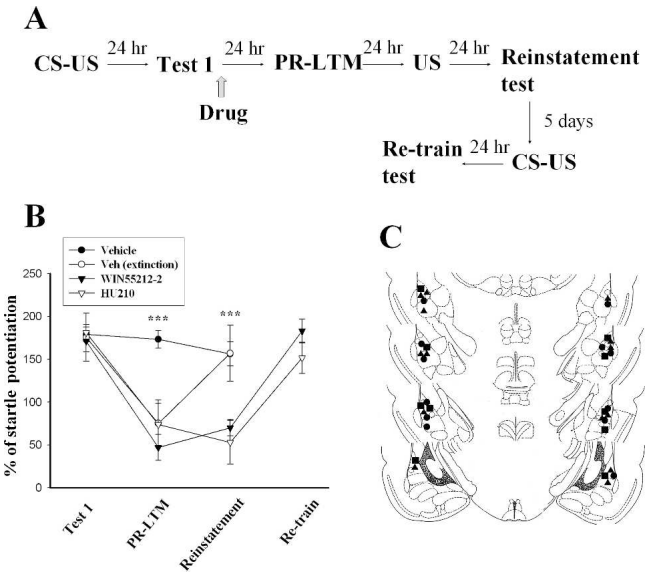

Figure 6. Retardation of reinstatement of fear memory by CB1 receptor agonists. (*A*) Behavioral procedure used for the experiments shown in *B*. (*B*) WIN (11 µg per side) or HU210 (10 µg per side) were infused into the amygdala bilaterally within 1 h after Test 1, which blocked reconsolidation. Amnesia resulting from CB1 agonist infusions did not show reinstatement with unconditioned foot-shocks. After retraining, the levels of startle potentiation in the WIN or HU210 rats were comparable with their Test 1. Vehicle extinction animals were trained and then exposed to three sessions of 10 CS-alone trials that led to extinction. Subsequent exposure of these rats to 10 foot-shocks reinstated the startle. ***$P < 0.001$ vs. vehicle. (*C*) Cannula tip placements from rats infused with vehicle (●), vehicle extinction (○), WIN (▲), or HU210 (■) in the experiments shown in *B*.

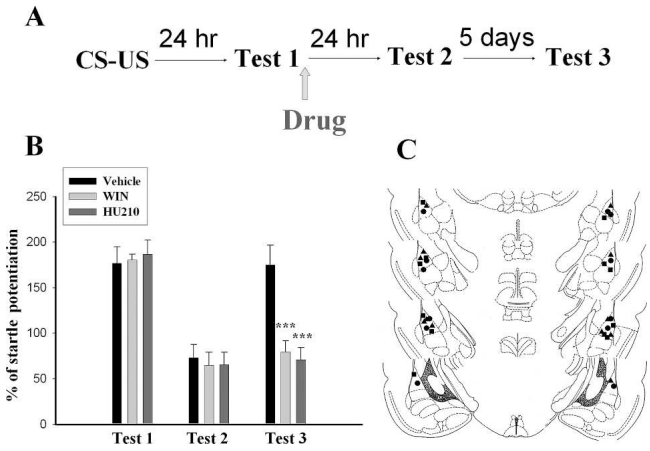

Figure 7. Retardation of spontaneous recovery by CB1 receptor agonists. (*A*) Behavioral procedure used in the experiment shown in *B*. (*B*) Animals were trained and then tested the next day. WIN (11 µg per side) or HU210 (10 µg per side) were infused into the amygdala bilaterally within 1 h after the test. Recovery of memory was assessed 7 d after training. Vehicle control rats were given extinction training to match the levels of startle in WIN group. ***$P < 0.001$ vs. vehicle. (*C*) Cannula tip placements from rats infused with vehicle (●), WIN (▲), or HU210 (■) in the experiments shown in *B*.

We assessed whether WIN produced an analgesic effect and affected baseline anxiety by measuring the shock reactivity and baseline startle, respectively, according to the methods described by Chhatwal et al. (2005). A separate group of conditioned rats was given an intra-amygdala infusion of WIN ($n = 5$) and 30 min later was presented with three shocks and 42 startle stimuli identical to those used in the above studies (0.6-mA, 0.5-sec foot-shocks, 95-dB startle stimuli). 3 d later, the same rats were returned to the startle box, injected with vehicle, and similarly tested. Figure 8 shows that there was no difference in shock sensitivity ($P = 0.32$) or baseline startle amplitude ($P = 0.67$) in rats given WIN or vehicle. Thus, intra-amygdala administration of WIN has no effect on pain sensitivity or baseline startle amplitude.

Discussion

In the present study, we have shown that post-test infusion of WIN or HU210 into the amygdala significantly impaired fear memory in a dose-dependent manner. The effects of WIN or HU210 could be reversed by the selective CB1 receptor antagonist and were no longer seen if the test was omitted. Re-exposing WIN-treated rats to the US failed to reinstate learned fear. In addition, the WIN-treated rats did not show spontaneous recovery. Finally, administration of CB1 agonists at the dose used in this study did not damage the amygdala neurons, induce apoptosis, or produce an obvious analgesic effect. Taken together, these results suggest that intra-amygdala infusion of CB1 receptor agonists could impair fear memory via an effect on reconsolidation.

Memory testing caused memory reactivation and initiated two potentially dissociable but opposite processes: reconsolidation and extinction (Nader et al. 2000; Myers and Davis 2002; Nader 2003; Suzuki et al. 2004). We have demonstrated that activation of the CB1 receptor in the amygdala impaired fear memory when CB1 agonists were administered immediately after test, but were not effective when administered without a test. In addition, no evidence of reinstatement and spontaneous recovery was found in WIN-treated animals. Based on the notion that original memory became labile and would not return after a specific block of reconsolidation (Duvarci and Nader 2004), reactivation-induced amnesia by CB1 agonists could be attributable to the block of reconsolidation. Extinction of conditioned fear in general was considered to be an inhibitory learning that prevented the expression of intact association rather than erasing it. If a memory deficit induced by CB1 agonists after memory reactivation was attributable to enhanced extinction, then re-exposing animals to the US prior to the test would restore its representation and reinstate the learned responses. In addition, testing animals at different time points after extinction should reveal a recovery of retention. A previous study by Chhatwal et al. (2005) has shown that systemic injection of a CB1 receptor antagonist prior to extinction training blocked extinction. Conversely, administration of the cannabinoid uptake inhibitor AM404 facilitated extinction in a dose-dependent manner. The difference between their results and ours is not clear, but could be due to different training protocols applied (extinction vs. memory testing) or the route of drug administration (systemic vs. intra-amygdala administration). Activation of CB1 receptors could facilitate extinction on one hand and block reconsolidation on the other.

Reinstatement and spontaneous recovery are signs of preservation of the original memory after extinction training. Theoretically, they could be used to judge whether a manipulation facilitates extinction as opposed to blocking reconsolidation. However, it should be cautioned that under certain circumstances if extinguishment of memory was caused by the erasure of original memory, then reinstatement and spontaneous recovery are not valid to differentiate between the facilitation of extinction and blocking of reconsolidation.

It is noted that intra-amygdala injection of a CB1 agonist immediately after the test impaired both PR-STM and PR-LTM, suggesting that CB1 agonists block a fast cascade of events nec-

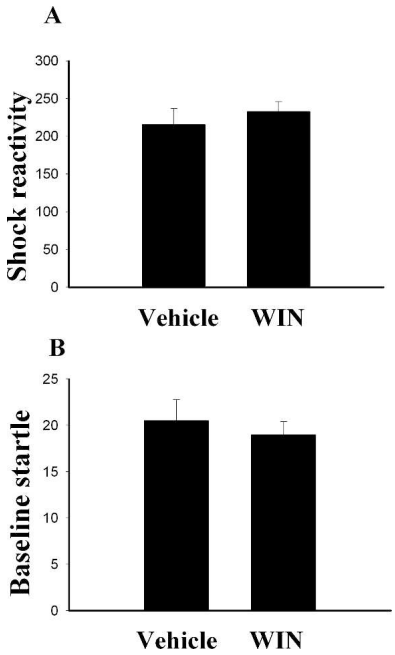

Figure 8. Effect of WIN on shock reactivity and baseline anxiety. Conditioned rats received an intra-amygdala infusion of WIN (11 µg/side, $n = 5$) and 30 min later were presented with three shocks and 42 startle stimuli (0.6-mA, 0.5-sec shocks, 95-dB noise-burst startle). 3 d later, the same rats were returned to the startle box, injected with vehicle, and similarly tested. (*A*) Shock reactivity represents the average response to three foot-shocks. (*B*) Baseline startle amplitude represents the average response to 42 startle stimuli.

essary for memory reconsolidation. It has been shown that PKA phosphorylation of S845 in GluR1 increased the peak open probability (Banke et al. 2000) of AMPA receptors as well as the surface reinsertion of GluR1 (Ehlers 2000). Furthermore, fear memory formation required the coupling of GluR1 and PKA by A-kinase anchoring proteins (AKAPs) through synapse-associated protein 97 kDa (SAP97) in the lateral amygdala (Moita et al. 2002). Thus, it is likely that activation of CB1 receptors negatively regulates adenylyl cyclase (Howlett et al. 1986; Bidaut-Russell et al. 1990), PKA, and phosphorylation of AMPA receptors, resulting in the retardation of formation and maintenance of STM. In this context, it has been shown recently that, using a low-intensity training protocol (1.3-mA US foot-shock), activation of PKA in the amygdala enhanced reconsolidation. In contrast, inhibition of PKA impaired reconsolidation when a high-intensity training protocol (2.0-mA US foot-shock) was applied (Tronson et al. 2006).

In summary, retrieval of memory would put it into a new vulnerable phase so that a reconsolidation blockade could lead to erasure of memory, not by inhibiting the expression of memory as extinction training did. Here, we have demonstrated that activation of CB1 receptors blocked reconsolidation, and rats given CB1 agonists immediately after a memory test failed to exhibit reinstatement and spontaneous recovery. Thus, CB1 agonists could be useful for the treatment of patients with post-traumatic stress disorders (PTSD) because the drug-treated patients may be less likely to relapse after a stressful experience.

Materials and Methods

Surgery

Rats anesthetized with sodium pentobarbital (50 mg/kg, i.p.) were mounted on a stereotaxic apparatus, and two cannulae made of 22-gauge stainless steel tubing were implanted bilaterally into the LA or BLA. The coordinates were AP −2.3 mm, ML ±4.5 mm, DV −7.0 mm according to Paxinos and Watson (1986). Only rats with cannula tips within the boundaries of LA and BLA were included in the data analysis. Rats were monitored and handled daily and were given 7 d to recover. WIN55212–2, HU210, and AM251 were obtained from Tocris Cookson Ltd. The drugs were dissolved in DMSO (50%) and administered bilaterally in a volume of 1 µL at a rate of 0.1 µL/min.

Behavioral apparatus and procedures

Rats were trained and tested in a stabilimeter device. A piezoelectric device mounted below the stabilimeter detects and transduces the motion of the cylinder produced by the whole body startle response of the rat (San Diego Instrument). The whole set-up was enclosed in a ventilated, sound-attenuating cabinet (length 38 cm, width 38 cm, height 55 cm). The acoustic startle stimulus was a 50-ms white noise at the intensity of 95 dB. The visual CS was a 3.7-sec light produced by an 8W fluorescent bulb attached to the back of the stabilimeter. The US was a 0.6-mA foot-shock with a duration of 0.5 sec.

Acclimation

On three consecutive days, rats were placed in the startle test boxes for 10 min and returned to their home cages.

Matching

On two consecutive days, rats were placed in the startle box and 3 min later presented with 10 startle stimuli at 2-min intertrial intervals (ITI). On the basis of their mean startle amplitudes in the second of these two sessions, rats were matched into groups with similar response levels.

Training

Rats were placed in the startle boxes and received 10 light–foot-shock pairings with an ITI of 2 min.

Test

24 h after training, rats were tested for fear-potentiated startle. This involved 10 startle-eliciting noise bursts presented alone (noise-alone trial) and 10 noise bursts presented 3.2 sec after onset of the 3.7-sec light (light–noise trials). The two trial types were presented in a balanced mixed order (ITI, 30 sec). The percentage of fear-potentiated startle was computed as follows: [(startle amplitude on CS-noise minus noise-alone trials) / (noise-alone trials)] × 100.

Reconsolidation

Rats were trained and memory was tested 24 h later (Test 1). Rats were infused with WIN55212–2, HU210, or vehicle within 1 h after termination of Test 1. A post-reactivation short-term memory (PR-STM) test was performed 4 h later, followed by a PR-LTM test 24 h after Test 1.

Reinstatement

Animals were trained according to the reconsolidation paradigm, returned to the testing chamber 24 h later, and presented with 10 foot-shocks. Animals underwent a test for memory reinstatement 24 h after foot-shock. 5 d later, rats were retrained with 10 light–foot-shock pairings, and the following day they were tested for the LTM of the retrained memory. A group of vehicle control rats was exposed to 30 trials of CS-alone extinction training to match the degree of startle reflex in WIN-treated animals.

Shock reactivity and baseline startle measurement

A group of conditioned rats was injected with WIN bilaterally into the amygdala, placed in the training box, and presented with three unpaired foot-shocks and 42 startle stimuli (0.6-mA, 0.5-sec shocks, 95-dB noise-burst startle). The same group of rats was returned to the same startle box 3 d later, injected with vehicle, and presented with identical foot-shocks and startle stimuli.

Histology

At the end of experiments, animals received an overdose of pentobarbital (100mg/kg), and the brains were removed from the skull and fixed in buffered 4% paraformaldehyde (pH 7.4) for 48 h. Brains were sectioned with a sliding MicroSlicer (DTK-1000, Ted Pella Inc.), and sections (40-µm thickness) were stained for Nissl bodies and DNA dye Hoechst 33,342 (bis-benzimide, Sigma). Nuclei were visualized using a fluorescence microscope.

Data analysis

Data were analyzed with ANOVA. A single-factor ANOVA and post hoc comparisons were used to analyze the dose-dependent effect of WIN55212–2 in blocking reconsolidation and the difference between the effect of drugs on STM and LTM. An unpaired *t*-test was used to analyze differences of startle reflex between the drug-treated and vehicle control groups. A paired *t*-test was used to analyze the difference in startle amplitude before and after a reminder shock in drug-treated rats (reinstatement experiments). All values in the text and figure legends are mean ±SEM.

Acknowledgments

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ORIGINAL INVESTIGATION

The cannabinoid receptor agonist WIN 55,212-2 facilitates the extinction of contextual fear memory and spatial memory in rats

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Abstract
Rationale Previous studies demonstrated that pharmacological blockade of CB1 cannabinoid receptors decreases the extinction of conditioned fear and spatial memory in rodents. However, the effects of CB1 cannabinoid receptor activation in this response remain unclear.
Objectives To evaluate the effects of the cannabinoid agonist WIN 55,212-2 (WIN) and the cannabinoid antagonist SR 147778 (SR) on the extinction of contextual fear memory in rats 24 h or 30 days after fear conditioning.
Methods For fear conditioning, rats were placed in the conditioning chamber for 3 min and received a 1-s electric foot shock (1.5 mA). Retrieval testing consisted of a 3-min exposure to the conditioning chamber and extinction training consisted of successive 9-min exposures at 24-h intervals. Rats were also evaluated in the open field and water maze reversal task.
Results The administration of SR (1.0 mg/kg, i.p.) and WIN (0.25 mg/kg, i.p.) before extinction training disrupted and facilitated, respectively, the extinction of 24 h contextual fear memory. These effects were not related to any disturbance in memory retrieval, unconditioned freezing expression, or locomotor activity. WIN (0.25 mg/kg, i.p.) also facilitated the extinction of 30-day-old contextual fear memory, while the prior administration of SR (0.2 mg/kg, i.p.) antagonized this response. The facilitative effect of WIN on memory extinction does not seem to be specific for contextual fear memory because it was also observed in the water maze reversal task.
Conclusions These results suggest cannabinoid receptor agonists as potential drugs to treat anxiety disorders related to the retrieval of aversive memories.

Keywords Fear conditioning · Spatial memory · Extinction · Cannabinoid · WIN 55,212-2 · SR 147778

Introduction

The endocannabinoid system has become a major focus in the search for novel therapies for many common mental disorders (Makriyannis et al. 2005) because an increasing amount of evidence suggests its important role in regulation of emotional states and cognitive processes (Terranova et al. 1996; Lichtman 2000; Marsicano et al. 2002; Takahashi et al. 2005). The physiological importance of the endocannabinoid system in emotional learning is supported by the dense expression of the CB1 cannabinoid receptors and the presence of endocannabinoids in brain regions known to be important for anxiety and aversive learning, including the amygdala and hippocampus (Herkenham et al. 1990; Di Marzo et al. 2000). Behavioral studies also provide compelling support for the involvement of the cannabinoid system in learning and memory processes. Cannabinoid agonists often induce cognitive impairments in rodents

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(Lichtman et al. 1995; Ferrari et al. 1999; Da Silva and Takahashi 2002; Varvel and Lichtman 2002; Pamplona and Takahashi 2006), whereas the antagonism of CB1 receptors generally enhances rodent performance in many memory tasks (Terranova et al. 1996; Reibaud et al. 1999; Lichtman 2000; Takahashi et al. 2005).

Special interest was shown in cannabinoid modulation of fear memories, as numerous similarities link the expression of fear and anxiety in humans suffering, such as phobias, posttraumatic stress disorder (PTSD), and other anxiety disorders, to the expression of conditioned fear in animals (Brewin and Holmes 2003). In fear conditioning paradigms, a conditioned stimulus (such as a context) is paired with an unconditioned stimulus (such as foot shock). When placed back in the context, the animal shows conditioned fear responses such as freezing. The duration of nonreinforced reexposures to the context is a crucial determinant of subsequent memory processing: brief reminders lead to reconsolidation, whereas longer reminders result in memory extinction, which tends to weaken the expression of the original memory (Suzuki et al. 2004). After this, a recent study at our laboratory demonstrated that the activation of CB1 cannabinoid receptors impairs the acquisition of contextual fear conditioning in rats with no effect on retrieval at all (Pamplona and Takahashi 2006). Furthermore, the endocannabinoids anandamide and 2-arachidonoylglycerol are released in the periaqueductal gray matter during stressful situations (Hohmann et al. 2005) and in the basolateral amygdala during the extinction of fear memories (Marsicano et al. 2002). Consequently, the genetic deletion of CB1 cannabinoid receptors results in a strong impairment of short-term and long-term extinction of conditioned fear, which was confirmed by the use of rimonabant, a selective CB1 cannabinoid receptor antagonist. The recent availability of SR 147778 (SR), a newly developed antagonist with high affinity and specificity for CB1 cannabinoid receptors (Rinaldi-Carmona et al. 2004), leads to the possibility of confirming and extending these previous findings observed with rimonabant (Rinaldi-Carmona et al. 1995). Moreover, in light of the fact that fear memories become increasingly resistant to extinction with age (Suzuki et al. 2004), it seems to be of interest to investigate whether the cannabinoid system may influence extinction of remote fear memories as well.

Therefore, the main objective of the present study was to examine whether the administration of the cannabinoid agonist WIN 55,212-2 (WIN) could facilitate the extinction of recent and/or remote contextual fear memory in rats. Further, we investigated the role of the CB1 cannabinoid receptors in the extinction processes using the newly developed selective CB1 cannabinoid receptor antagonist SR. The water maze reversal task was also used to investigate whether the influence of the cannabinoid system on memory extinction would generalize to extinction of spatial memory in rats.

Materials and methods

Animals

Male adult Wistar rats (3 months old) bred and raised in the animal facility of the Department of Pharmacology of Universidade Federal de Santa Catarina (UFSC) were used. The animals were kept in collective plastic cages (five to six rats per cage) with food and water available ad libitum. They were maintained in a room under controlled temperature (23±2°C) and a 12:12-h light/dark cycle (lights on at 7:00 A.M.). Each behavioral test was conducted during the light phase of the cycle (between 8:00 A.M. and 5:00 P.M.) using independent experimental groups consisting of seven to ten animals per group. All the experimental procedures were performed according to the guidelines on animal care of the UFSC Ethics Committee on the Use of Animals, which follows the "principles of laboratory animal care" from NIH.

Drugs and treatment

WIN [*R*-(+)-(2,3-dihydro-5-methyl-3-[{4-morpholinyl}methyl] pyrol [1,2,3-de-]-1,4-benzoxazin-6-yl)(1-naphthalenyl) methanone mesylate] (Tocris, USA) and SR [5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-ethyl-*N*-(1-piperidinyl)-1*H*-pyrazole-3-carboxamide] (Sanofi-Aventis, France) were dissolved in 0.9% NaCl (saline) with 10% dimethylsulfoxide plus 0.1% Tween 80. The control solution consisted of a drug vehicle. All drug doses, selected according to previous literature (Lichtman et al. 1995; Chhatwal et al. 2005; Takahashi et al. 2005; Pamplona and Takahashi 2006), were administered intraperitoneally in a volume of 0.2 ml/100 g of body weight. WIN and SR were administered 30 and 20 min, respectively, before behavioral test, except in experiment 3 in which SR was administered 20 min before WIN.

Behavioral procedures

Fear conditioning

The conditioning chamber consisted of a modified shuttle box (Automatic Reflex Conditioner model 7531, Ugo Basile, Italy) made of gray opaque Plexiglas. One of the compartments (22×22×25 cm) of the chamber was used for tone and contextual fear conditioning. Contextual conditioning tests were conducted in the chamber and tone conditioning tests were conducted in a different context, consisting of a

transparent glass cage (30×30×30 cm). The experiments were carried out in a sound-attenuated room under low intensity light (10 lx) and a microvideo camera was mounted at the top of the chamber, allowing the experimenter to observe the rats on a monitor placed in an adjacent room. Tone and contextual fear conditioning were performed with modifications from a procedure previously described by Corodimas et al. (2000). For contextual fear conditioning, rats were placed in the conditioning chamber for 3 min and received a 1-s electric foot shock (1.5 mA), after which they were kept for an additional minute in the chamber before being returned to their home cages. For tone fear conditioning, the rats were placed in the conditioning chamber, and after 3 min a sound (1,000 Hz, 80 dB) was presented for 10 s that coterminated with a 1-s electric foot shock (1.5 mA). The rats were kept for an additional minute in the chamber before being returned to their home cages. Independent groups of animals were used in each experiment. Freezing, defined as a stereotyped crouching position with complete immobility of the animal, except for the movements necessary for breathing, was used as a memory index during the subsequent nonreinforced reexposures to the context or tone (Blanchard and Blanchard 1969; Fanselow 1980). Freezing time was recorded with stopwatches by an experienced observer who was blind to the conditions of the treatment. The same observer recorded freezing in all the experiments to avoid individual variabilities and obtain more reliable results.

Experiment 1: effects of cannabinoid receptor ligands on extinction of recent contextual fear memory Successive long exposures to the conditioning chamber were used to test the effects of cannabinoids on short-term (within-exposure) and long-term (between-exposure) extinction of conditioned fear. For this, 24 h after contextual fear conditioning, the animals were exposed to the conditioning chamber for 9 min and the freezing behavior was evaluated. This extinction procedure was executed three times at 24-h intervals to give an index of long-term extinction of conditioned freezing. Moreover, the percentage of freezing during the first extinction session was used to investigate any possible within-session effects of drug treatment (Quirk et al. 2000; Marsicano et al. 2002; Fernandez-Espejo 2003). The animals were treated with WIN (0.25, 1.25, or 2.50 mg/kg, i.p.), SR (0.2, 1.0, or 2.0 mg/kg, i.p.) or control solution before each extinction session.

Experiment 2: effects of cannabinoid receptor ligands on retrieval of contextual fear memory Contrasting with the extinction procedure, a single short exposure to the conditioning chamber was used to test the effect of cannabinoids on retrieval of conditioned fear with minimal interference of within-session extinction (McKay et al. 2002). For this, 24 h after contextual fear conditioning, the animals were exposed for 3 min to the conditioning chamber and the freezing behavior was evaluated (Sorg et al. 2004). The animals were treated with WIN (0.25, 1.25, or 2.50 mg/kg, i.p.), SR (0.2, 1.0, or 2.0 mg/kg, i.p.), or control solution before being reexposed to the conditioning chamber.

Experiment 3: effects of the cannabinoid agonist WIN on extinction of remote contextual fear memory Thirty days after being simultaneously subjected to tone and contextual fear conditioning, the animals were exposed to the conditioning chamber for 9 min for freezing evaluation. Because aversive memories become increasingly resistant to disruption with age (Suzuki et al. 2004), this extinction procedure was executed five times at 24-h intervals.To investigate whether the effects of WIN on extinction of contextual fear memory in rats were related to the activation of CB1 cannabinoid receptors, the animals were treated with SR (0.2 mg/kg, i.p.) or control solution (i.p.), and 20 min later they were injected with WIN (0.25 mg/kg, i.p.) or control solution (i.p.) 30 min before each extinction session. Also, to investigate whether the WIN effects were selective to the memory that was extinguished, 24 and 48 h after the end of the extinction protocol (fifth day), the rats were tested in a drug-free state for retrieval of the tone and contextual fear conditioning, respectively. For retrieval of tone fear conditioning, they were placed in a different context (transparent acrylic cage, 30×30×30 cm) and three 1-min sound presentations were made with 1-min intervals. Twenty-four hours after, the rats were exposed to the conditioning chamber for 3 min for retrieval of the contextual fear conditioning. Freezing behavior was evaluated during each test.

Unconditioned freezing behavior

Experiment 4: effects of cannabinoid receptor ligands on the expression of unconditioned freezing behavior Rats were placed in the conditioning chamber for 3 min and after this period they received a 1-s electric foot shock (1.5 mA), after which they were kept for one additional minute in the chamber before being returned to their home cages. Twenty-four hours after, they were treated with WIN (0.25, 1.25, or 2.50 mg/kg, i.p.), SR (0.2, 1.0, or 2.0 mg/kg, i.p.), or control solution and exposed for 3 min to a new context (transparent glass cage, 30×30×30 cm) for evaluation of unconditioned freezing behavior.

Open field

The open field apparatus was made of white painted wood with a white 100×100 cm floor (divided into 25 squares of 20×20 cm) and 40-cm-high white walls.

Experiment 5: effects of cannabinoid receptor ligands on locomotor activity Rats were injected with WIN (0.25, 1.25, or 2.50 mg/kg, i.p.), SR (0.2, 1.0, or 2.0 mg/kg, i.p.), or control solution and placed in the center of the open field for 3 min of free exploration. The number of squares crossed was registered and used as an index of locomotor activity.

Water maze reversal task

To test whether the effects of the activation and blockade of CB1 cannabinoid receptors on extinction of contextual fear memory could be generalized to another hippocampus-dependent task with different sensory, motivational, and performance demands, the rats were tested in the water maze reversal task previously described by Varvel and Lichtman (2002). The water maze consisted of a circular swimming pool made of black painted fiberglass (inside diameter 1.70 m and 0.8 m high, filled to a depth of 0.6 m with water maintained at 25°C). The target platform (10×10 cm) was made of transparent Plexiglas and was submerged 1–1.5 cm beneath the surface of the water. Starting points for the animals were marked on the outside of the maze as north (N), south (S), east (E), and west (W). The platform was located in the center of the northeast quadrant at a point 35 cm from the wall of the maze. Four distant visual cues (55×55 cm) were placed on the walls of the experimental room to allow spatial orientation by the animals.

Experiment 6: effects of cannabinoid receptor ligands on extinction of spatial memory in rats Rats were assigned to two training sessions separated by an interval of 24 h, each of which consisted of six consecutive trials with the platform remaining in the fixed position. The animals were left in one of the aforementioned starting points facing the wall of the maze and were allowed to swim freely to the platform. If an animal did not find the platform during a period of 60 s, it was gently guided to the platform's location and allowed to remain for 10 s on it before being removed from the water maze for 20 s and subsequently placed at the next starting point. Twenty-four hours after the second training session, rats received WIN (0.25 mg/kg, i.p.), SR (1.0 mg/kg, i.p.), or control solution (i.p.) and were subjected to a reversal task in which the platform was moved to the opposite side of the tank (center of the southwest quadrant). The starting points and the intertrial intervals were identical to those of the training sessions. The time the animals spent reaching the platform (escape latency) was used as the learning/memory index in both the training sessions and the reversal task.

Data analysis

The statistical comparison of results was carried out using one-way ANOVA with treatment as the independent factor or two-way ANOVA with treatment and trials (repeated measure) as independent factors. After significant ANOVAs, differences between groups were evaluated by post hoc Duncan's test. The accepted level of significance for the tests was $p \leq 0.05$. All statistical analyses were performed using the Statistica® 6.0 software package (StatSoft, USA).

Results

Experiment 1: effects of cannabinoid receptor ligands on extinction of recent contextual fear memory The effects of SR (0.2, 1.0, or 2.0 mg/kg, i.p.) on extinction of contextual fear memory evaluated 24 h after fear conditioning are given in Fig. 1a. Two-way ANOVA revealed a significant effect for treatment [$F(3,26)=5.18$, $p<0.01$] and trials [$F(2,52)=11.67$, $p<0.0001$], but no treatment × trial interaction. Post hoc comparisons indicated that the extinction protocol of 3 days significantly decreased the freezing time across successive reexposures of the control group to the conditioning chamber ($p \leq 0.05$, second and third trials compared to the first). The intermediate dose of SR (1.0 mg/kg, i.p.) disrupted the extinction of contextual fear memory as indicated by an increased freezing time compared to the control group ($p \leq 0.05$).

The effects of WIN (0.25, 1.25, or 2.50 mg/kg, i.p.) on extinction of contextual fear memory, evaluated 24 h after fear conditioning, are given in Fig. 1b. Two-way ANOVA revealed a significant effects for treatment [$F(3,29)=6.84$, $p<0.001$] and trials [$F(2,58)=17.31$, $p<0.00001$], but no treatment × trial interaction. Post hoc comparisons indicated that the control group presented a partial extinction of contextual fear conditioning after three reexposures to the conditioning chamber ($p \leq 0.05$, third compared to the first exposure). The administration of WIN promoted a dose-dependent effect on the extinction process. The group treated with the lowest dose of WIN (0.25 mg/kg, i.p.) exhibited a decreased freezing time during the first 9-min exposure compared to the control group ($p \leq 0.05$) and it underwent partial extinction on the third trial ($p \leq 0.05$, compared to the first), suggesting a facilitative effect of this dose in the extinction of contextual fear conditioning. In contrast, the higher dose of WIN (2.50 mg/kg, i.p.) disrupted the extinction of conditioned fear as evidenced by the lack of reduction in the freezing time across the trials and an increased freezing time compared to the group treated with the lowest dose of WIN (0.25 mg/kg, i.p.). The intermediate dose of WIN (1.25 mg/kg, i.p.) exhibited a

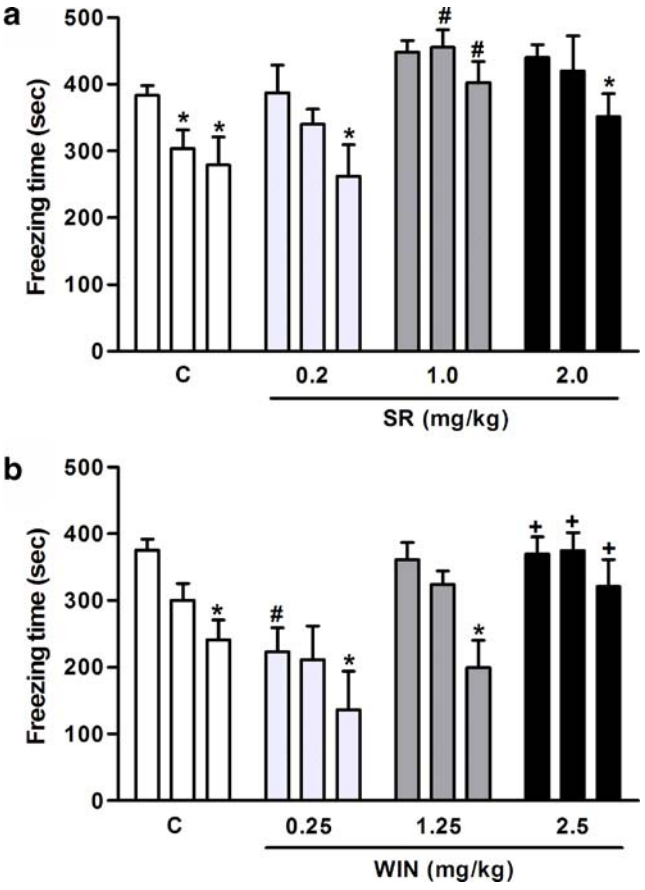

Fig. 1 Effects of the selective CB1 cannabinoid receptor antagonist SR (0.2, 1.0 or 2.0 mg/kg, i.p.) and cannabinoid agonist WIN (0.25, 1.25, or 2.50 mg/kg, i.p.) on the extinction of recent contextual fear memory in rats. Data are expressed as mean±SEM of the time spent freezing expressed by SR-treated rats (**a**) and WIN-treated rats (**b**) during three 9-min exposures to the conditioning chamber with 24-h intervals (each *bar* represents the data of one session). *Asterisk*: $p \leq 0.05$ compared to the first session of the corresponding group. *Number sign*: $p \leq 0.05$ compared to the control group during the corresponding session. *Plus sign*: $p \leq 0.05$ compared to the group treated with the lowest dose of WIN (0.25 mg/kg, i.p.) during the corresponding session (Duncan's post hoc test). (Control $n=8$, SR 0.2 $n=7$, SR 1.0 $n=8$, and SR 2.0 $n=7$) (Control $n=9$, WIN 0.25 $n=7$, WIN 1.25 $n=7$, and WIN 2.5 $n=10$)

profile of extinction similar to that of the control group. As reduction of freezing time in the group treated with WIN (0.25 mg/kg, i.p.) might suggest that WIN affected the retrieval of memory and not its extinction, the results of the first extinction session (9 min) were reanalyzed in 3-min bins. Further analysis of freezing levels showed no significant difference during the first 3-min bin [$F(3,29)=2.57$, $p=0.07$], but a marked treatment effect was noted in the second [$F(3,29)=8.1$, $p=0.0004$] and third [$F(3, 29)=6.06$, $p=0.002$] 3-min bins. Post hoc comparisons revealed that WIN (0.25 mg/kg, i.p.) did not influence memory retrieval (first 3 min), but facilitated short-term extinction, reducing the freezing time in the second and third 3-min bins compared to the control group ($p \leq 0.05$ for both). This result was confirmed in experiment 2.

Experiment 2: effects of cannabinoid receptor ligands on retrieval of contextual fear memory The effects of SR (0.2, 1.0, or 2.0 mg/kg, i.p.) and WIN (0.25, 1.25, 2.5 mg/kg, i.p.) on the retrieval of contextual fear memory are given in subpanels a and b in Fig. 2, respectively. One-way ANOVA of the results of each experiment revealed a nonsignificant effect for treatment with SR [$F(3,32)=0.38$, $p=0.77$] or WIN [$F(3,28)=1.56$, $p=0.22$].

Experiment 3: effects of the cannabinoid agonist WIN on extinction of remote contextual fear memory The effects of WIN (0.25 mg/kg, i.p.) on extinction of 30-day-old contextual fear memory in rats are given in Fig. 3. Two-way ANOVA revealed a significant effect for treatment [$F(2,29)=13.62$, $p<0.0001$] and trials [$F(4,116)=18.02$, $p<0.00001$], but no treatment × trial interaction. Post hoc comparisons indicated that the administration of WIN (0.25 mg/kg, i.p.) significantly decreased the freezing time compared to the control group ($p \leq 0.05$), suggesting a facilitative effect of WIN on the extinction of remote contextual fear memory. Moreover, a per se ineffective dose of SR (0.2 mg/kg, i.p.) antagonized the effect of WIN (0.25 mg/kg, i.p.) ($p \leq 0.05$), suggesting that it was related to the activation of the CB1 cannabinoid receptors.

As illustrated in Fig. 3b, to investigate whether the WIN effects were selective toward the memory that was extinguished, 24 and 48 h after the end of the extinction protocol (fifth day), the rats were tested in a drug-free state for retrieval of the tone and context fear conditioning. One-way ANOVA revealed no significant treatment effect on the freezing time during tone presentation [$F(2,29)=0.71$, $p=0.50$], demonstrating that the tone-shock association was unaffected by the extinction of contextual fear memory (Fig. 3b). However, one-way ANOVA revealed significant treatment effect on the freezing time during reexposure to the context [$F(2,29)=4.48$, $p<0.005$]. Indeed, 48 h after the end of the fifth extinction session, the control group continued to express pronounced freezing behavior when reexposed to the conditioning chamber, whereas the time spent freezing by drug-free rats previously given WIN was significantly shortened ($p \leq 0.05$) (Fig. 3b). This latter effect was antagonized by SR (0.2 mg/kg, i.p.), emphasizing the involvement of CB1 cannabinoid receptors on the facilitative effects of WIN on extinction of remote contextual fear memory (Fig. 3b).

Experiment 4: effects of cannabinoid receptor ligands on the expression of unconditioned freezing behavior The effects of WIN (0.25, 1.25, or 2.5 mg/kg, i.p.) or SR (0.2, 1.0, or 2.0 mg/kg, i.p.) on the expression of unconditioned

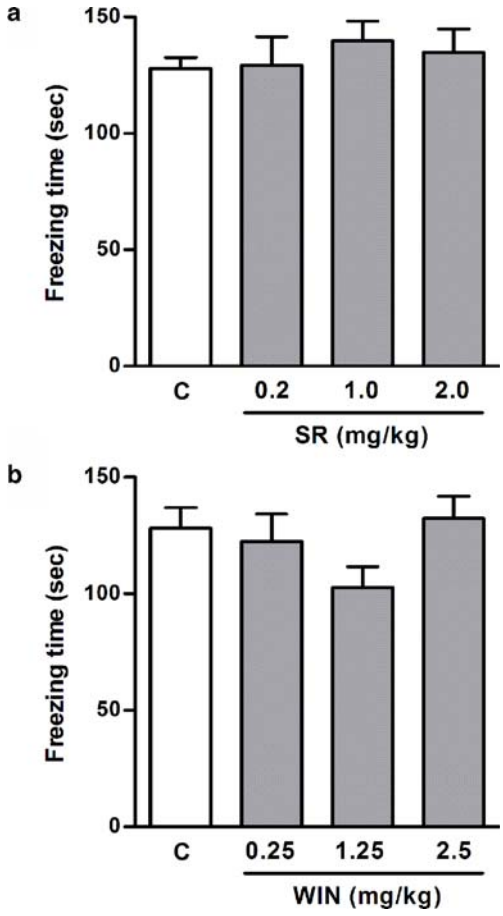

Fig. 2 Effects of the selective CB1 cannabinoid receptor antagonist SR (0.2, 1.0, or 2.0 mg/kg, i.p.) and cannabinoid agonist WIN (0.25, 1.25, or 2.50 mg/kg, i.p.) on the retrieval of recent contextual fear memory in rats. Data are expressed as mean±SEM of the time spent freezing expressed by SR-treated rats (**a**) and WIN-treated rats (**b**) during a 3-min exposure to the conditioning chamber. (Control n=9, SR 0.2 n=8, SR 1.0 n=10, and SR 2.0 n=9) (Control n=10, WIN 0.25 n=7, WIN 1.25 n=7, and WIN 2.5 n=8)

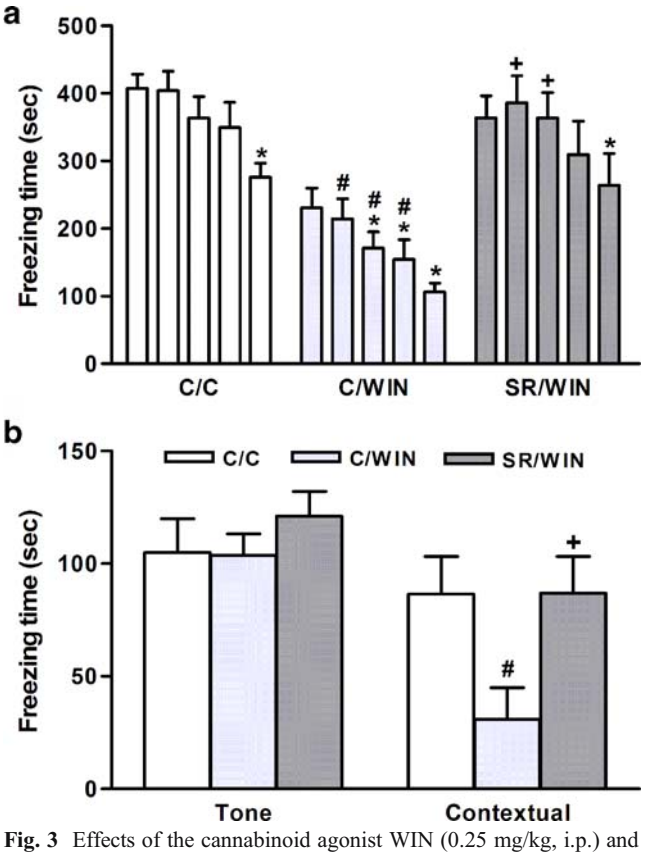

Fig. 3 Effects of the cannabinoid agonist WIN (0.25 mg/kg, i.p.) and pretreatment with the selective CB1 cannabinoid receptor antagonist SR (0.2 mg/kg, i.p.) on extinction of remote contextual fear memory in rats. The animals received one injection of SR or control solution (**c**) followed by one injection of WIN or control solution before each extinction session. **a** Mean±SEM of the time spent freezing expressed by the animals during five 9-min exposures to the conditioning chamber with 24-h intervals (each *bar* represents the data of one session). **b** (*Left*) Mean±SEM of the time spent freezing during a 3-min drug-free tone presentation, 24 h after the extinction of contextual fear conditioning; (*right*) mean±SEM of the time spent freezing during a 3-min drug-free exposure to the conditioning chamber, 48 h after the extinction of contextual fear conditioning. *Asterisk*: $p \leq 0.05$ compared to the first session of the corresponding group. *Number sign*: $p \leq 0.05$ compared to the C/C group during the corresponding session. *Plus sign*: $p \leq 0.05$ compared to the C/WIN group during the corresponding session (Duncan's post hoc test). (C/C n=9, C/WIN n=12, and SR/WIN n=11)

freezing behavior in rats are summarized in Table 1. One-way ANOVA revealed no significant effect for treatment on the time of unconditioned freezing [$F(6,52)=1.02$, $p=0.42$].

Experiment 5: effects of cannabinoid receptor ligands on locomotor activity The effects of WIN (0.25, 1.25, or 2.5 mg/kg, i.p.) or SR (0.2, 1.0, or 2.0 mg/kg, i.p.) on the locomotor activity of rats in the open field test are summarized in Table 1. One-way ANOVA revealed no significant effect for treatment on the number of squares crossed [$F(6,49)=1.81$, $p=0.12$].

Experiment 6: effects of cannabinoid receptor ligands on extinction of spatial memory in rats The effects of WIN (0.25 mg/kg, i.p.) or SR (1.0 mg/kg, i.p.) on rats subjected to the water maze reversal task are illustrated in Fig. 4. Two-way ANOVA revealed a significant effect of trials on escape latency during the two training sessions [day 1 $F(5,105)=24.63$, $p<0.00001$; day 2 $F(5,105)=9.45$, $p<0.00001$] with no difference between groups (Fig. 4a). Two-way ANOVA for the data of the reversal task revealed a significant effect for trials [$F(5,105)=17.16$, $p<0.00001$] and treatment × trial interaction [$F(10,105)=2.61$, $p<0.005$]. Post hoc comparisons indicated that WIN-treated (0.25 mg/kg, i.p.) animals showed decreased escape latencies in the first trial of the water maze reversal task, whereas SR-treated (1.0 mg/kg, i.p.) animals showed increased escape latencies in the second trial of the water maze reversal task compared to the control group ($p \leq 0.05$) (Fig. 4b).

Table 1 Effects of WIN (0.25, 1.25, or 2.5 mg/kg, i.p.) and SR (0.2, 1.0, or 2.0 mg/kg, i.p.) on unconditioned freezing and open field behavior

Treatment (mg/kg)	Unconditioned freezing (s)	Number of samples	No. of squares crossed	Number of samples
Control	31.9±5.9	11	63±6	13
SR 0.2	36.7±10.4	8	70±4	7
SR 1.0	41.1±8.4	8	69±4	7
SR 2.0	25.5±5.0	8	74±9	7
WIN 0.25	35.6±8.1	8	58±3	7
WIN 1.25	18.0±7.1	8	59±4	7
WIN 2.5	23.7±10.3	8	50±6	8

Discussion

The present findings confirm and extend those of previous studies demonstrating that the disruption of CB1 cannabinoid receptor signaling decreases the extinction of conditioned fear in rodents. More importantly, our results suggest that the extinction of contextual fear memory in rats may be facilitated by the cannabinoid agonist WIN, and that this response was antagonized by the new selective CB1 cannabinoid receptor antagonist SR. Furthermore, the present facilitative effects of WIN on memory extinction in rats cannot be attributed to alterations in memory retrieval or sensorimotor deficits and does not seem to be specific for conditioned fear memory because it was also observed for spatial memory.

In the present study, we present evidence that the administration of the new selective CB1 cannabinoid receptor antagonist SR (1.0–2.0 mg/kg, i.p.) disrupts the extinction of contextual fear memory in rats evaluated 24 h after fear conditioning. Our findings are in accordance with those of recent studies showing that CB1 knockout mice and mice and rats treated with the selective CB1 cannabinoid receptor antagonist rimonabant exhibit a pronounced deficit in the extinction of conditioned fear (Marsicano et al. 2002; Suzuki et al. 2004; Chhatwal et al. 2005). Furthermore, the present results demonstrate that a low dose of the cannabinoid agonist WIN (0.25 mg/kg, i.p.) may facilitate the extinction of conditioned fear in rats. This last finding extends to fear memory the previous results of Parker et al. (2004), showing that low doses of Δ^9–tetrahydrocannabinol and cannabidiol promote extinction of conditioned place preference in rats. It is interesting to note that we failed to show any enhancement of memory extinction using higher doses of WIN (1.25–2.5 mg/kg, i.p). Accordingly, WIN (5.0 mg/kg, i.p.) did not facilitate the extinction of fear-potentiated startle (Chhatwal et al. 2005). A potential discrepancy in the present study is the notion that rats treated with WIN (0.25 mg/kg, i.p) and showing reduced freezing during the first extinction session might have experienced some kind of impairment in fear memory retrieval. However, in keeping with the present results and previous reports (Lichtman 2000; Da Silva and Takahashi 2002; Marsicano et al. 2002; Varvel and Lichtman 2002; Chhatwal et al. 2005; Varvel et al. 2005; Pamplona and Takahashi 2006), neither WIN nor SR modified the performance in memory retrieval tasks, suggesting that the present effects of pharmacological manipulations of the cannabinoid system are specific for memory extinction. It could also be speculated that the present results may reflect some combination of sensorimotor deficits induced by drug treatment, rather than the facilitation of memory extinction. However, freezing behavior can hardly account for the present results because neither SR nor WIN altered the number of squares crossed in the open field test or the amount of unconditioned freezing expressed by rats.

The effects of the cannabinoid system on the extinction of remote aversive memories in rats were also investigated.

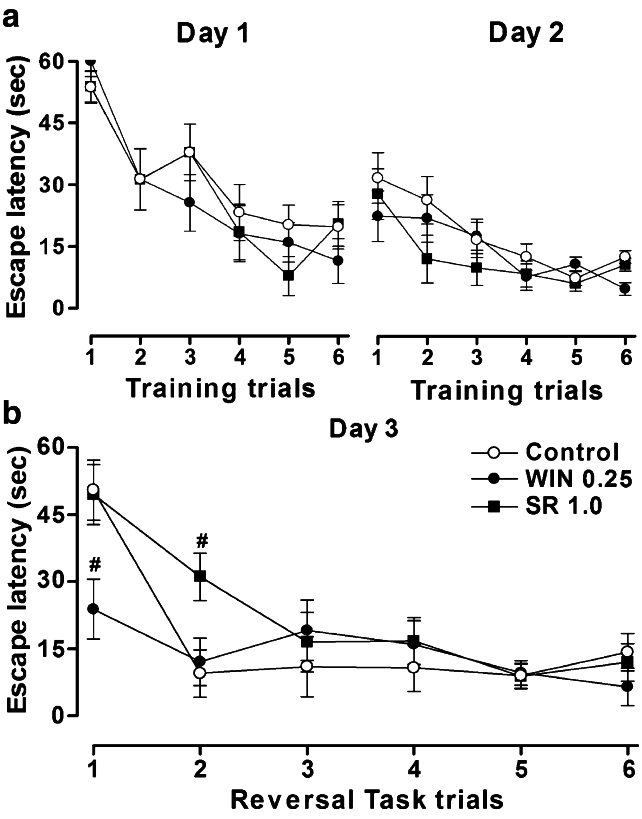

Fig. 4 Effects of the selective CB1 cannabinoid receptor antagonist SR (1.0 mg/kg, i.p.) and cannabinoid agonist WIN (0.25 mg/kg, i.p.) on the performance of rats in the water maze reversal task. **a** The animals were trained to find a submerged platform in a fixed position during six trials on two consecutive days. **b** One day later, they received drug treatment and were tested in the reversal task in which the platform location was changed to the opposite quadrant of the water maze. Each *point* represents the mean±SEM of the escape latency (s) to reach the platform location. *Number sign*: $p \leq 0.05$ compared to the control group during the corresponding trial (Duncan's post hoc test). (Control n=8, WIN n=8, and SR n=8)

As previously reported by Suzuki et al. (2004), the age of a specific memory is strongly determinant of the ease of its disruption. Corroborating a previous study (Suzuki et al. 2004), the remote contextual fear memory (30 days) was harder to extinguish than a recent one (24 h) because it required a protocol of five extinction sessions to exhibit a partial extinction. Nevertheless, the cannabinoid agonist WIN (0.25 mg/kg, i.p.) also facilitated the extinction of remote aversive memories through the activation of CB1 cannabinoid receptors. Furthermore, the effect of WIN was selective for the memories, which were extinguished and had long-lasting consequences, which clearly emphasizes the long-term facilitative effects of WIN on extinction of conditioned fear.

In addition, our findings also suggest that the endocannabinoid system modulates the extinction of spatial memory in rats evaluated in the water maze because the administration of SR (1.0 mg/kg, i.p.) and WIN (0.25 mg/kg, i.p.) transiently disrupted and improved, respectively, the performance of rats in the water maze reversal task. It must be conceded that the Wistar rats employed have poor visual capabilities, which may partially compromise these results. Nevertheless, our results are in accordance with those of earlier studies that demonstrate deficits in the extinction of previously learned spatial information in mice as a consequence of CB1 cannabinoid receptor deletion or blockade (Varvel and Lichtman 2002; Varvel et al. 2005).

In conclusion, the present results reinforce those of previous studies demonstrating that the disruption of CB1 cannabinoid receptor signaling impairs the extinction of both conditioned fear and spatial memory in rodents. More importantly, our results suggest that the extinction of contextual fear memory and spatial memory in rats may be facilitated by the cannabinoid agonist WIN with long-lasting effects. Because it was demonstrated that a drug that facilitates extinction of conditioned fear in laboratory animals may also be utilized with success in humans (Walker et al. 2002; Ressler et al. 2004), pharmacotherapies directed at the endocannabinoid system may represent a viable approach to the treatment of a variety of psychiatric disorders related to the retrieval of fear memories, including panic, phobias, and PTSD.

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RESEARCH PAPER

5-HT$_{1A}$ receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats

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Background and purpose: Cannabidiol (CBD) is a non-psychotomimetic compound from *Cannabis sativa* which induces anxiolytic- and antipsychotic-like effects in rodents. These effects could be mediated by facilitation of the endocannabinoid system or by the activation of 5-HT$_{1A}$ receptors. As either of these mechanisms could promote adaptation to inescapable stress, the aim of the present work was to test the hypothesis that CBD would attenuate the autonomic and behavioural consequences of restraint stress (RS). We also investigated if the responses to CBD depended on activation of 5-HT$_{1A}$ receptors.
Experimental approach: Male Wistar rats received i.p. injections of vehicle or CBD (1, 10 or 20 mg kg^{-1}) and 30 min later were submitted to 60 min of restraint where their cardiovascular responses were recorded. The protocol of the second experiment was similar to the first one except that animals received i.p. injections of the 5-HT$_{1A}$ receptor antagonist WAY100635 (0.1 mg kg^{-1}) before CBD treatment and exposure to restraint. 24 h later they were also tested in the elevated plus-maze (EPM), an animal model of anxiety.
Key results: Exposure to RS increased blood pressure and heart rate and induced an anxiogenic response in the EPM 24 h later. These effects were attenuated by CBD. WAY100635 by itself did not change the cardiovascular and anxiogenic response to RS, but blocked the effects of CBD.
Conclusion and implications: The results suggest that CBD can attenuate acute autonomic responses to stress and its delayed emotional consequences by facilitating 5-HT$_{1A}$ receptor-mediated neurotransmission.
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Keywords: cannabinoids; cardiovascular system; elevated plus-maze; 5-HT$_{1A}$ receptor

Abbreviations: CBD, cannabidiol; EPM, elevated plus-maze; HR, heart rate; MAP, mean arterial pressure; THC, Δ^9-tetrahydrocannabinol

Introduction

Marijuana (from *Cannabis sativa*) is one of the most widely abused drugs in the world. In humans, it elicits subjective changes that include euphoria, heightened sensitivity to external stimuli and relaxation (Martin *et al.*, 1991; Compton *et al.*, 1992; Johns, 2001). The major constituent of cannabis is Δ^9-tetrahydrocannabinol (THC) and this is thought to be the main ingredient responsible for its psychoactive properties (Mechoulam, 1970; Mechoulam *et al.*, 1970; Ilan *et al.*, 2005). The discovery of specific binding sites for THC led to the discovery of the cannabinoid receptors (Devane *et al.*, 1988; Matsuda *et al.*, 1990; Munro *et al.*, 1993) and, so far, two sub-types of the cannabinoid receptor have been identified, CB_1 and CB_2 (Pertwee, 2005; nomenclature follows Alexander *et al.*, 2008). The activation of CB_1 receptors by THC is thought to account for most of the central effects of cannabis (Huestis *et al.*, 2001). Anandamide and 2-arachidonoyl glycerol, referred to as endocannabinoids, are the major endogenous agonists of the CB_1 receptor (Di Marzo *et al.*, 1998; Piomelli, 2003).

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Cannabidiol (CBD), another cannabinoid generally found in relatively high concentrations in cannabis, exhibits a somewhat different pharmacology compared with THC (Mechoulam *et al*., 2002). CBD attenuates the psychotomimetic and anxiogenic effects of THC in humans (Karniol *et al*., 1974; Zuardi *et al*., 1982). Moreover, systemic administration of CBD induced antipsychotic (Zuardi *et al*., 1991; Zuardi *et al*., 2006) and anxiolytic-like effects (Guimaraes *et al*., 1990; Resstel *et al*., 2006).

The mechanism of action of CBD is not fully understood. It has a low affinity for cannabinoid receptors (Petitet *et al*., 1998; Thomas *et al*., 1998) but can block the reuptake of anandamide (Bisogno *et al*., 2001) and its metabolism by the enzyme, fatty acid amide hydrolase (FAAH), (Watanabe *et al*., 1998; Di Marzo *et al*., 1999; Mechoulam and Hanus, 2002; Mechoulam *et al*., 2002). Moreover, CBD may possess agonistic properties at 5-HT_{1A} receptors (Russo *et al*., 2005). Although there are contradictory results, several studies indicate that activation of these receptors can induce anxiolytic-like effects and mediate adaptation to stress (Blier and de Montigny, 1994; Blier and Ward, 2003; Joca *et al*., 2003; Joca *et al*., 2007).

Acute restraint is an uncontrollable stress situation that produces endocrine and autonomic responses characterized by increases in glucocorticoids levels, blood pressure and heart rate (HR) (Tavares and Correa, 2006; Hsu *et al*., 2007). These responses are accompanied by activation of several brain structures (Pacak and Palkovits, 2001). In addition to physiological responses, animals submitted to restraint also develop behavioural changes reflected, for example, in reduced exploratory activity in an open field 24 h after stress (Kennett *et al*., 1985a; 1987; Mechiel Korte and De Boer, 2003), increased immobility in a forced swimming test (Sevgi *et al*., 2006) and reduced exploration of the open arms of an elevated plus-maze (EPM) (Guimaraes *et al*., 1993; Padovan and Guimaraes, 2000). These stress-induced behavioural changes can be attenuated by systemic or intracerebral administration of anxiolytic and antidepressant drugs (Kennett *et al*., 1985a; 1987; Guimaraes *et al*., 1993; Padovan and Guimaraes, 2000; Mechiel Korte and De Boer, 2003). A possible effect of CBD on these changes, however, has not yet been investigated. Therefore, in the present work we tested the hypothesis that systemic administration of CBD would attenuate the acute physiological changes and the behavioural consequences of restraint stress. We also evaluated the involvement of 5-HT_{1A} receptors in the effects of CBD in this model.

Methods

Animals

The Institution's Animal Ethics Committee approved housing conditions and experimental procedures for animals. Male Wistar rats weighing 230–250 g were used. The animals were provided by our local Animal farm facility. After arriving at the Animal Care Unit of the Department of Pharmacology, School of Medicine of Ribeirão Preto, University of Sao Paulo, the animals were kept in groups of four per cage for 48 h. After this adaptation period they are housed individually in plastic cages with free access to food and water under a 12 h light/dark cycle (lights on at 06:30 h) for a further 24 h period before being subjected to the surgical procedure (see below).

Surgical preparation

Twenty-four hours before being submitted to restraint stress, animals had a polyethylene catheter implanted into the femoral artery under anaesthesia (tribromoethanol, 250 mg kg^{-1} i.p.), for the recording of arterial blood pressure and HR. The catheter was exposed on the dorsum of the animals and attached to the skin, allowing arterial pressure recordings from conscious animals.

Acute restraint

Experiment 1 In the morning period (between 7 a.m. and 12 a.m.), the animals were transferred to the experimental room in their home box. Mean arterial pressure (MAP) and HR were recorded using an HP-7754A amplifier (Hewlett Packard, Palo Alto, CA, USA) connected to a signal acquisition board (Biopac M-100, Goleta, CA, USA) and computer processed. After a few minutes of baseline recording, rats received a single i.p. injection of one of the following drugs: vehicle or CBD (1, 10 or 20 mg kg^{-1}). Thirty minutes later they were submitted to a 60 min restraint period in a small plastic cylindrical restraining tube (diameter = 6.5 cm and length = 15 cm). After the restraint period the animals returned to their cages. Each animal was submitted to only one session of restraint to prevent the development of stress tolerance (Guimaraes *et al*., 1993).

Experiment 2 Based on the results obtained in the first experiment, we chose the dose of 10 mg kg^{-1} of CBD to use in this second study. The protocol was similar to the first experiment except that before the restraint period the animals received, first, an i.p. injection of vehicle or WAY (0.1 mg kg^{-1}) followed, 30 min later, by a second injection of vehicle or CBD (10 mg kg^{-1}). As in experiment 1, 30 min after the last injection the animals were restrained for 60 min. One day later they were tested in the EPM. We also had a general control group of unrestrained animals treated with the saline+CBD vehicle that were tested 24 h later in the EPM.

The EPM test

The EPM test was conducted as described before (Padovan and Guimaraes, 2000). Briefly, the apparatus consisted of two opposite open arms (50 × 10 cm) crossed at a right angle by two arms of the same dimensions enclosed by 40 cm high walls with no roof. The maze was located 50 cm above the floor. Rodents naturally avoid the open arms of the EPM and anxiolytic compounds typically increase the exploration of these arms without changing the number of enclosed-arm entries (Pellow *et al*., 1985; Carobrez and Bertoglio, 2005). The EPM was cleaned and dried before each session and the Ethovision software (Version 1.9, Noldus, Netherlands) was employed for behavioural analysis.

Data analysis

Mean arterial pressure and HR values were continuously recorded for 10 min before the 60 min restraint stress period.

Table 1 Basal values of the MAP and HR in vehicle (control), CBD and WAY100635 treated rats

Group		*MAP (mmHg)*	*HR (bpm)*
Control	$n = 6$	105 ± 2	338 ± 13
CBD 1 mg	$n = 5$	103 ± 3	363 ± 14
CBD 10 mg	$n = 5$	100 ± 3	349 ± 11
CBD 20 mg	$n = 5$	97 ± 3	369 ± 9
WAY100635	$n = 5$	99 ± 2	371 ± 14
		$F_{4,25} = 1.5$, $P > 0.05$	$F_{4,25} = 1.3$, $P > 0.05$

The values in the table represent the means ± SE. One-way ANOVA.
CBD, cannabidiol; HR, heart rate; MAP, mean arterial pressure.

Data were expressed as means ± SEM of MAP or HR changes (respectively Δ MAP and Δ HR) sampled at 5 min intervals. Points sampled during the 10 min before restraint were used as control baseline value. MAP and HR changes were analysed using two-way ANOVA with treatment as independent factor and time as repeated measurement factor. When interaction between the factors was observed, groups were compared using one-way ANOVA followed by Bonferroni's *post hoc* test.

The per cent of entries (100 × open/total entries) and time spent in the open arms (100 × open/open + enclosed) of the EPM were calculated for each rat. These data, together with the number of enclosed arm entries, were analysed by one-way ANOVA followed by Bonferroni's *post hoc* test. Values of $P < 0.05$ were taken as showing statistically significant differences between means.

Drugs

The following drugs were used: CBD (THC Pharma, Frankfurt, Germany): 1, 10 or 20 mg kg^{-1}, suspended in polyoxyethylenesorbitan monooleate (Tween 80) 2%-saline (Resstel *et al.*, 2006), WAY100635 (WAY, Sigma, St. Louis, MO, USA): 0.1 mg kg^{-1} dissolved in saline (Kaster *et al.*, 2005) and tribromoethanol (Aldrich, St. Louis, MO, USA). The solutions were prepared immediately before use and injected intraperitoneally in a volume of 1 mL kg^{-1}. The appropriate vehicles were used in each experiment.

Results

Experiment 1

Effects of CBD on cardiovascular responses to acute restraint Systemic injection of CBD (1, 10 and 20 mg kg^{-1}) did not affect baseline blood pressure ($F_{4,25} = 1.2$, $P > 0.05$) or HR ($F_{4,25} = 0.9$, $P > 0.05$) values when compared with vehicle control ($n = 6$, Table 1). As represented in Figure 1, acute restraint induced a marked and sustained increase of HR and MAP during the 60 min test. There were significant effects of treatment (MAP: $F_{3,240} = 93.5$, $P < 0.001$; HR: $F_{3,240} = 123$, $P < 0.001$), time (MAP: $F_{14,240} = 83.6$, $P < 0.001$; HR: $F_{14,240} = 27.9$, $P < 0.001$) and treatment versus time interaction (MAP: $F_{42,240} = 5.1$, $P < 0.01$; HR: $F_{42,240} = 7.9$, $P < 0.01$).

Cannabidiol decreased the stress-induced cardiovascular responses at doses of 10 and 20 mg kg^{-1} (HR, $F_{3,16} = 19.9$, $P < 0.001$, MAP, $F_{3,16} = 14.6$, $P < 0.001$). The dose of 1 mg kg^{-1} was also able to attenuate HR responses ($P < 0.05$). A dose-dependency was demonstrated by nonlinear regression analysis and showed a significant correlation between doses and attenuation of the increased MAP ($r^2 = 0.82$, df = 13, $P < 0.01$) and HR ($r^2 = 0.73$, df = 13, $P < 0.01$) (Fig. 2).

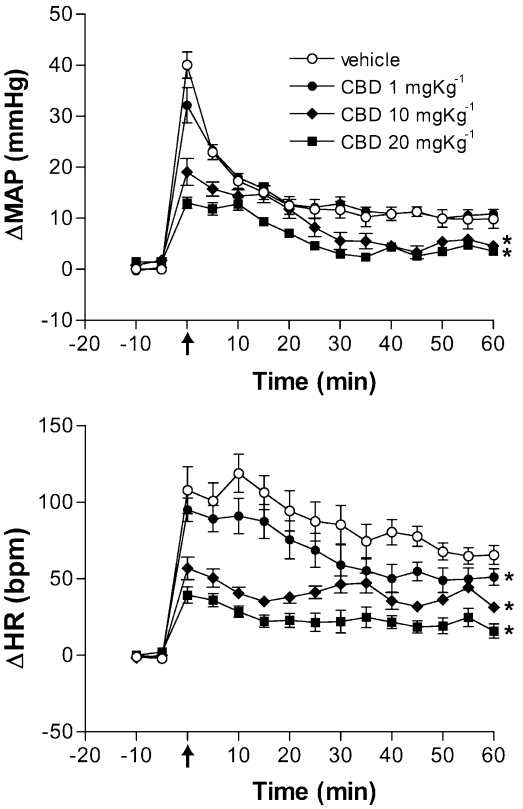

Figure 1 Effects of different doses of cannabidiol (CBD, 1, 10 or 20 mg kg^{-1}, $n = 5$ per group) or vehicle (Tween 80 2%-saline, 1 mL kg^{-1}, $n = 6$) on changes in mean arterial pressure (ΔMAP) and heart rate (ΔHR) of animals submitted to 60 min of restraint stress. The arrow indicates the beginning of the restraint period. Data shown represent the means ± SEM. *$P < 0.05$, compared with vehicle group; ANOVA followed by Bonferroni's *post hoc* test.

Experiment 2a

Effects of WAY100635 on CBD effects on acute cardiovascular responses to restraint WAY ($n = 5$) did not affect baseline values of blood pressure ($F_{4,25} = 1.2$, $P > 0.05$) or HR ($F_{4,25} = 0.9$, $P > 0.05$) compared with CBD (10 mg kg^{-1}, $n = 6$) and vehicle ($n = 6$, Table 1).

There were significant effects of restraint (MAP: $F_{3,270} = 35.95$, $P < 0.001$; HR: $F_{3,270} = 104.3$, $P < 0.001$), time (MAP: $F_{14,270} = 59.66$, $P < 0.001$; HR: $F_{14,270} = 66.4$, $P < 0.001$) and treatment versus time interaction (MAP: $F_{42,270} = 2.1$, $P < 0.01$; HR: $F_{42,270} = 1.9$, $P < 0.01$) (Fig. 3).

The decrease of the cardiovascular responses by systemic administration of CBD (10 mg kg^{-1}) was prevented by WAY (MAP: $F_{1,135} = 139.5$, $P < 0.001$; HR: $F_{1,135} = 290.3$, $P < 0.001$) (Fig. 3). The latter drug, by itself, had no effect on cardiovascular responses to restraint (MAP: $F_{1,135} = 1.3$, $P > 0.05$; HR: $F_{1,135} = 1.1$, $P > 0.05$) (Fig. 3).

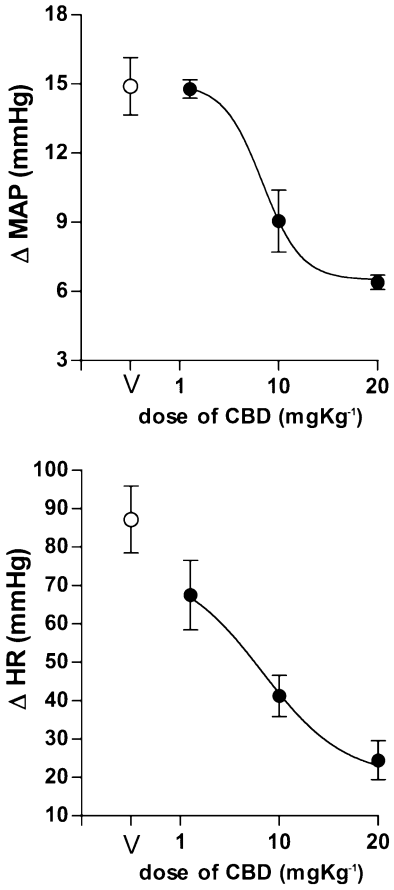

Figure 2 Mean arterial pressure (ΔMAP) and heart rate (ΔHR) changes in response to the injection of increasing doses of cannabidiol (CBD, 1, 10, 20 mg kg^{-1}, $n = 5$/group) in rats. V: vehicle (Tween 80 2%-saline, 1 mL kg^{-1}, $n = 6$). Dose- effect curves were generated by nonlinear regression analysis. Data shown represent the means ± SEM of the variation of MAP and HR during the 60 min of acute restraint.

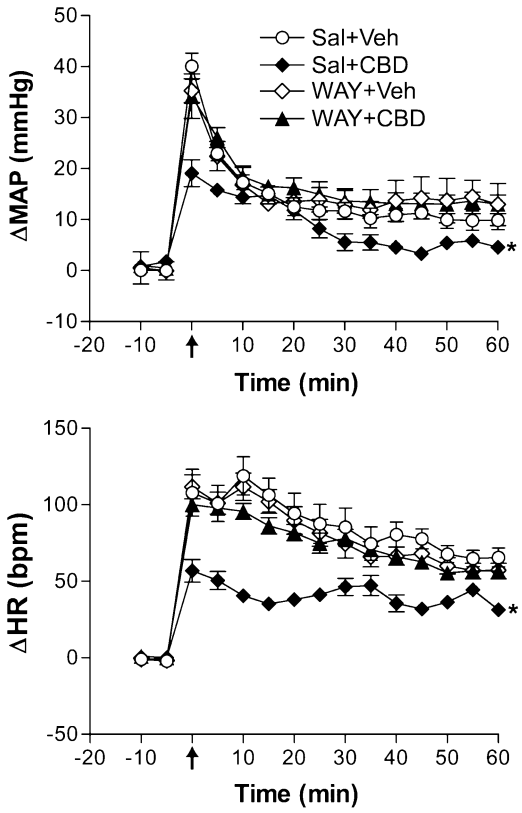

Figure 3 Effects of pre-treatment with of saline (Sal) or WAY100635 (WAY, 0.1 mg kg^{-1}) followed by second injection of vehicle (Veh, Tween 80 2%-saline) or cannabidiol (CBD, 10 mg kg^{-1}) immediately before a 60 min restraint period on increase in the mean arterial pressure (ΔMAP) and heart rate (ΔHR) induced by restraint stress. The arrow indicates the beginning of the restraint period. Data shown represent the mean ± SEM of five to six animals. *$P < 0.05$, compared with vehicle group; ANOVA followed by Bonferroni's *post hoc* test.

Typical experimental recordings showing the effects of CBD and WAY on cardiovascular responses observed during acute restraint can be seen in Figure 4.

Experiment 2b

Effects of CBD and WAY100635 on delayed behavioural consequences in the EPM induced by restraint Acute restraint induced a significant decrease in the percentage of open arm entries ($F_{3,25} = 7.72$, $P < 0.001$) compared with unrestrained controls ($n = 6$), when tested 24 h after stress (Fig. 5). CBD administration in restrained rats ($n = 6$) increased the percentage of open arm entries ($P < 0.001$, Fig. 5) compared with controls (vehicle-treated restrained animals, $n = 6$). This effect was prevented by pre-administration of WAY ($P < 0.001$, $n = 6$). No effect was found in animals treated only with WAY ($P > 0.05$, $n = 6$) (Fig. 5). There was also no effect on the percentage time spent in the open arms and in the number of enclosed arm entries ($P > 0.05$).

Discussion

The present results showed that (i) CBD reduced the pressor and tachycardic responses to restraint stress, in a dose-dependent manner; (ii) CBD attenuated the increased anxiety behaviour caused by a previous exposure to restraint; and (iii) effects of CBD on cardiovascular and behavioural responses to restraint can be blocked by WAY100635, a 5-HT_{1A} receptor antagonist.

Acute exposure to restraint stress has been shown to evoke several physiological and behavioural changes (Tavares and Correa, 2006; Hsu *et al.*, 2007) and, as expected, in the present study animals exhibited significant increases in MAP and HR during restraint. Moreover, they showed decreased exploration of the open arms of the EPM 24 h after restraint stress. These delayed behavioural consequences of acute restraint stress have been described in several models, including the open field and EPM (Kennett *et al.*, 1985a; Guimaraes *et al.*, 1993; Padovan and Guimaraes, 2000; Mechiel Korte and De Boer, 2003), and are sensitive to systemic and intra-cerebral injection of anxiolytic and antidepressant drugs (Kennett *et al.*, 1985a; Kennett *et al.*, 1987; Guimaraes *et al.*, 1993; Padovan and Guimaraes, 2000; Mechiel Korte and De Boer, 2003).

Cannabidiol did not induce any significant change in basal MAP and HR which agrees with the reported lack of significant cardiovascular effects of this drug (McQueen *et al.*, 2004;

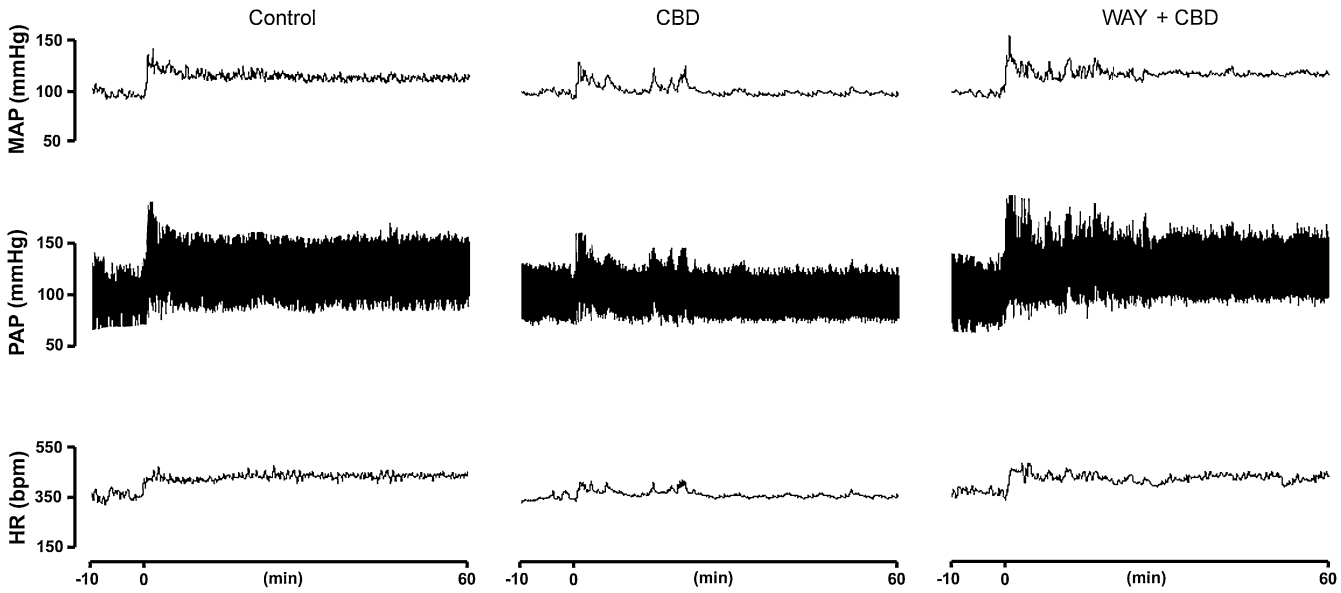

Figure 4 Mean arterial pressure (MAP), pulsatile arterial pressure (PAP) and heart rate (HR) individual recordings showing the cardiovascular changes evoked by acute restraint in animals treated with vehicle (control, Tween 80 2%-saline), cannabidiol (CBD) or cannabidiol after WAY100635 (WAY+CBD). The restraint period started at time 0.

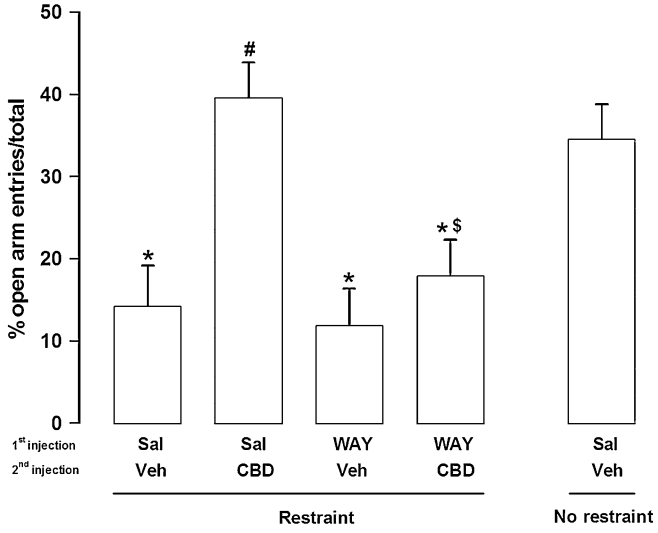

Figure 5 Effects in the elevated plus-maze (EPM) of a first systemic injection of saline (Sal) or WAY100635 (WAY, 0.1 mg kg^{-1}) followed by a second injection of vehicle (Veh, Tween 80 2%-saline) or cannabidiol (CBD, 10 mg kg^{-1}) immediately before a 60 min restraint period ($n = 6$ per group). A non-stressed group (no-restraint) that received i.p. injections of saline followed by vehicle was used as general control. The EPM test was performed 24 h after the restraint period. Data represent the mean (±SEM) per cent of open arm entries. $^{*}P < 0.05$, compared with control group; $^{\#}P < 0.05$, compared with restraint-vehicle group; $^{\$}P < 0.05$, compared with restraint-CBD group; ANOVA followed by Bonferroni's *post hoc* test.

Resstel *et al.*, 2006). At low doses, it also does not interfere with memory and learning processes (Lichtman *et al.*, 1995; Fadda *et al.*, 2004; 2006). It is unlikely, therefore, that the attenuation of the cardiovascular and delayed behavioural responses to restraint depend on direct cardiovascular effects or memory impairment induced by the drug, but rather on an attenuation of the emotional response to stress. In agreement with this proposal, acute administration of CBD has been shown to induce anxiolytic-like effects in several animal models, including the EPM, Vogel conflict test and contextual fear conditioning (Guimaraes *et al.*, 1990; Onaivi *et al.*, 1990; Moreira *et al.*, 2006; Resstel *et al.*, 2006). The effective doses of CBD in these previous studies were, in general, similar to ours. In the study by Guimaraes *et al.* (1990), however, CBD produced an inverted U-shaped dose-response curve, with the highest dose (20 mg kg^{-1}) being ineffective. The reasons for this difference are unknown, but may depend on the model used (Calabrese, 2008). As the EPM test is based on exploratory activity, it could be more prone to interference of non-specific drug effects that affects this parameter (Calabrese, 2008). In addition, at least for classical anxiolytics such as diazepam, the EPM is more sensitive than other models such as the Vogel conflict test (see Calabrese, 2008).

The mechanisms of the anxiolytic and anti-stress effects of CBD are not clear. It has a low affinity for cannabinoid receptors (Petitet *et al.*, 1998; Thomas *et al.*, 1998) although, under certain circumstances, it may act as an antagonist of CB_1- and CB_2-receptor agonists (Pertwee *et al.*, 2002). CBD could also block the reuptake and metabolism of anandamide, facilitating endocannabinoid-mediated neurotransmission (Watanabe *et al.*, 1998; Di Marzo *et al.*, 1999; Bisogno *et al.*, 2001; Mechoulam *et al.*, 2002). However, several effects of CBD have been shown to be independent of the endocannabinoid system (Hayakawa *et al.*, 2007). More recently, Russo *et al.* (2005) reported that CBD can displace the 5-HT agonist [^{3}H]8-OH-DPAT from cloned human 5-HT_{1A} receptors expressed in Chinese hamster ovary cultured cells. Moreover, using signal transduction studies, this work also showed that CBD can act as an agonist at these receptors. The observation that CBD has agonistic properties at 5-HT_{1A} receptors has been supported by *in vivo* studies where the neuroprotective and anti-oxidative effects induced by CBD were blocked by pre-treatment with the 5-HT_{1A} antagonist WAY100135 (Mishima

et al., 2005; Hayakawa *et al*., 2007). Our results corroborate these findings, by showing that the stress-attenuating effects induced by CBD were prevented by systemic pretreatment with WAY100635, a selective antagonist at these receptors. In agreement with our results, a recent study from our laboratory showed that CBD interacts with 5-HT_{1A} receptors in the dorsolateral periaqueductal gray to produce anxiolytic-like effects in the EPM (Campos and Guimaraes, 2008).

5-HT_{1A} receptors are widely distributed in the brain, especially in structures traditionally related to stress and anxiety, such as the raphé nuclei, hippocampus, prefrontal cortex, amygdala and hypothalamus (Chalmers and Watson, 1991). Although the role of 5-HT in anxiety is still a matter of intense debate (Millan, 2003), several pieces of evidence suggest that activation of 5-HT_{1A} receptors facilitates adaptation to stress (Kostowski *et al*., 1992; Dekeyne *et al*., 2000; Tsuji *et al*., 2000; Blier and Ward, 2003; Joca *et al*., 2003; Kagamiishi *et al*., 2003; Rioja *et al*., 2004; Joca *et al*., 2007). For example, pre-administration of a 5-HT_{1A} agonist before an acute immobilization period blocked the stress-induced anxiogenic effect observed in an elevated T-maze test performed 24 h later (Rioja *et al*., 2004). Similar results were reported by Tsuji *et al*. (2000), who observed that administration of 5-HT_{1A} receptor agonists before restraint stress attenuated, in a dose-dependent manner, the development of stress-induced anxiogenic effect observed 24 h later in the hole-board test of anxiety. Data from 5-HT_{1A} receptor knockout mice give further support to this hypothesis, as these animals display anxiogenic-like behaviour in different paradigms (Zhuang *et al*., 1999; Tsetsenis *et al*., 2007). It is proposed that the lack of this receptor would promote a bias in the processing of threatening cues which could render the animal more susceptible to the development of behavioural consequences of stress (Tsetsenis *et al*., 2007). On the other hand, facilitation of this neurotransmission would mediate adaptation to stress (Graeff *et al*., 1996). This suggestion is supported by results showing that behavioural adaptation to stress is accompanied by sensitization of 5-HT_{1A}-mediated neurotransmission (Kennett *et al*., 1985b; 1987; Samad and Haleem, 2007) and that an increased expression of this receptor in the brain is associated with reduced anxiety-like behaviour (Kusserow *et al*., 2004). Therefore, these studies support the possibility that activation of 5-HT_{1A} receptors protects animals against various emotional changes caused by stressful stimuli, perhaps by facilitating mechanisms involved in the ability to cope with the stressful situation.

The exact mechanism through which 5-HT_{1A} agonists induce their anxiolytic activity remains unclear. 5-HT_{1A} receptors are located presynaptically (somatodendritic autoreceptors) in 5-hydroxytryptaminergic cell bodies in the raphé nuclei of the brain stem and postsynaptically, predominantly in limbic structures such as the hippocampus and the hypothalamus (Verge *et al*., 1985; 1986; Chalmers and Watson, 1991). It is still controversial whether the anxiolytic-like effects induced by acute systemic administration of 5-HT_{1A} agonists are due to the activation of the pre- or the post-synaptic receptors (File *et al*., 1996; Lopez-Rubalcava, 1996). Moreover, depending on the structure where post-synaptic 5-HT_{1A} receptors are located, their activation may lead to anxiolytic or anxiogenic-like effects (Graeff *et al*., 1996; Zangrossi *et al*., 2001). Therefore, the exact mechanism of action of 5-HT1A agonists as anxiolytic compounds is complex and still warrants further investigation.

In conclusion, the present findings indicate that CBD, by activating 5-HT_{1A} receptors, can attenuate physiological and behavioural responses to restraint stress. This finding raises the possibility that CBD could be useful for treating psychiatric disorders thought to involve impairment of stress-coping mechanisms, such as depression and post-traumatic stress disorder.

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Conflicts of interest

The authors state no conflict of interest.

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Behavioral/Systems/Cognitive

Cannabinoid Receptor Activation in the Basolateral Amygdala Blocks the Effects of Stress on the Conditioning and Extinction of Inhibitory Avoidance

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Despite the efficacy of behavior therapy for human anxiety disorders, extinction-like treatments require repeated cue exposures and are vulnerable to reversal by a number of environmental factors, particularly stress. The endocannabinoid system has recently emerged as important in the regulation of extinction learning and in the regulation of the hypothalamic–pituitary–adrenal axis. Here, we aimed to examine the involvement of the cannabinoid CB_1 receptor in the basolateral amygdala (BLA) in inhibitory avoidance (IA) conditioning and extinction and to test whether cannabinoid activation would reverse the effects of stress on these memory processes. The synthetic full agonist of the CB_1/CB_2 receptor WIN55,212-2 [*R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl) methanone monomethanesulfonate] (5 μg/0.5 μl) microinjected into the BLA had no effect on IA conditioning or extinction by itself. However, microinjecting WIN55,212-2 into the BLA before exposing the rats to a stressor reversed the enhancing effects of the stressor on IA conditioning and its impairing effects on IA extinction. Importantly, WIN55,212-2 microinjected into the BLA reduced stress-induced elevations in corticosterone levels. Control experiments demonstrated the following: (1) the effects of WIN55,212-2 could not be attributed to sensorimotor deficits, because these parameters seemed unchanged by WIN55,212-2 microinjected into the BLA; and (2) the CB_1 receptor in the BLA is crucially involved in the extinction of IA, because the CB_1 receptor antagonist AM251 [*N*-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide] (6 ng/0.5 μl) microinjected into the BLA significantly blocked extinction. Together, our findings may support a wide therapeutic application for cannabinoids in the treatment of conditions associated with the inappropriate retention of aversive memories and stress-related disorders.

Introduction

Fear inhibition is most often studied through a procedure in which a previously fear-conditioned organism is exposed to a fear-eliciting cue in the absence of any aversive event. This procedure results in a decline in conditioned fear responses that is attributed to a process called extinction (Myers and Davis, 2007).

Despite the efficacy of behavior therapy for human anxiety disorders, extinction-like treatments require repeated cue exposures and are vulnerable to reversal by a number of environmental factors, particularly stress. We recently showed (Akirav and Maroun, 2007) that 30 min of exposure to the elevated platform stressor disrupts the extinction of both auditory and contextual fear conditioning. Others have reported that stress reduces cued fear extinction (Shumake et al., 2005; Izquierdo et al., 2006; Maren and Chang, 2006) or impairs its recall (Maren and Chang, 2006; Miracle et al., 2006; Garcia et al., 2008). In parallel, exposure to stress facilitates the initial fear learning, thus further enhancing the fear response (Shors et al., 1992; Cordero et al., 2003).

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Manipulation of the endogenous cannabinoid system has become a major focus of current search for novel therapeutics to treat many common mental illnesses, including anxiety disorders, depression, and drug addiction (Porter and Felder, 2001; Kathuria et al., 2003). It is generally appreciated that the recreational use of cannabinoids is related to their positive modulatory effects on brain-rewarding processes along with their ability to positively influence emotional states and remove stress responses to environmental stimuli (Gardner and Vorel, 1998). Indeed, the potential therapeutic value of cannabinoid modulation is underscored by the dense expression of the cannabinoid CB_1 receptor in regions known to be significant for anxiety and emotional learning, particularly the basolateral amygdala (BLA) (Katona et al., 2001; Haller et al., 2002).

The endocannabinoid system has recently emerged as important in the regulation of extinction learning (Marsicano et al., 2002; Varvel and Lichtman, 2002; Suzuki et al., 2004; de Oliveira Alvares et al., 2005) and of the hypothalamic–pituitary–adrenal (HPA) axis and its end product corticosterone (CORT) (Patel et al., 2004; Cota, 2008; Steiner and Wotjak, 2008). Studies so far suggest that environmental stress and CB_1 receptor activity interact in the regulation of the HPA axis and that the augmentation of endocannabinoid signaling can suppress stress-responsive systems (Patel et al., 2004; Cota, 2008; Steiner and Wotjak, 2008).

Our main goal was to test whether cannabinoid activation in the BLA would inhibit stress-induced alterations in inhibitory

avoidance (IA) conditioning and extinction and to examine the possible association with the HPA axis. To that end, we examined the following: (1) the effects of administering cannabinoid receptor agonist into the BLA on the conditioning and extinction of IA, (2) whether cannabinoid activation in the BLA would reverse the effects of stress on IA conditioning and extinction, and (3) whether cannabinoid activation in the BLA would affect plasma CORT levels.

Materials and Methods

Subjects. A total of 434 male Sprague Dawley rats (~60 d old, 250–300 g) were used for the experiments. Animals were caged individually at 22 ± 2°C under 12 h light/dark cycles. Rats had access to water and laboratory rodent chow *ad libitum*. The experiments were approved by the University of Haifa Ethics and Animal Care Committee, and adequate measures were taken to minimize pain or discomfort in accordance with the guidelines laid down by the National Institutes of Health in the United States regarding the care and use of animals for experimental procedures.

Drug treatments. Three drugs were investigated: the synthetic CB_1/CB_2 receptor agonist WIN55,212-2 [*R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol[1,2,3-de]-1,4- benzoxazin-6-yl)(1-naphthalenyl) methanone monomethanesulfonate] (WIN); an inhibitor of endocannabinoid reuptake and breakdown, AM404 [*N*-(4-hydroxyphenyl)-arachidonamide]; and the CB_1 receptor antagonist AM251 [*N*-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide] (Tocris Bioscience). Each drug was initially dissolved in dimethylsulfoxide (DMSO) and further diluted with saline (0.9% NaCl).

The final DMSO concentration was <7%. This was also used as the vehicle. The final concentration of DMSO did not affect performance in the inhibitory avoidance task. Drug concentrations are based on reports in the literature (Martin et al., 1999; Chhatwal et al., 2005; de Oliveira Alvares et al., 2005; Moreira et al., 2007; Pamplona et al., 2008) and our preliminary results. For microinjection, WIN55,212-2 was used at 2.5 μg/0.5 μl, 5 μg/0.5 μl, or 10 μg/0.5 μl. AM404 was used at 200 ng/0.5 μl or 800 ng/0.5 μl, and AM251 was used at 6 ng/0.5 μl. For intraperitoneal administration, WIN 55,212-2 was used at 0.25 mg/kg.

Cannulation and drug microinjection. Rats were anesthetized with 4.8 ml/kg Equithesin (2.12% w/v $MgSO_4$ 10% ethanol, 39.1% v/v propylene glycol, 0.98% w/v sodium pentobarbital, and 4.2% w/v chloral hydrate), restrained in a stereotactic apparatus (Stoelting), and implanted bilaterally with a stainless steel guide cannula (23 gauge, thin walled) aimed at the BLA (anteroposterior, −3 mm; lateral, ±5 mm; ventral, −6.7 mm). The cannulae were set in place with acrylic dental cement and secured by two skull screws. A stylus was placed in the guide cannula to prevent clogging. Animals were allowed 1 week to recuperate before being subjected to experimental manipulations.

For microinjection, the stylus was removed from the guide cannula, and a 28 gauge injection cannula, extending 1.0 mm from the tip of the guide cannula, was inserted. The injection cannula was connected via polyethylene PE20 tubing to a Hamilton microsyringe driven by a microinfusion pump (CMA/100; Carnegie Medicine). Microinjection was performed bilaterally in a 0.5 μl volume per side delivered over 1 min. The injection cannula was left in position for an additional 30 s before withdrawal to minimize dragging of the injected liquid along the injection tract.

Light–dark inhibitory avoidance. Animals were placed in an inhibitory avoidance apparatus with a metal grid floor. The apparatus was divided into a light side and a dark side, and the rats were placed in the light side, facing the left rear corner of the box.

For conditioning (Cond), when the rats crossed over to the dark side of the box (with four paws on the grid), they received a 2 s, 0.7 mA scrambled footshock. After administration of the footshock, the opening between the two sides of the box was blocked, and the rats remained in the dark side for an additional 60 s, after which they were removed back to the home cage.

For extinction, rats were submitted to a non-reinforced test trial every 24 h for three days (Ext1–Ext3), beginning 24 h after conditioning. Each rat was placed in the light side of the box, and the time elapsed until it crossed over to the dark side (i.e., latency) was measured. If, after 180 s, the rat did not cross over on its own, the experimenter gently guided it to the dark side. The opening between the two sides of the shuttle was then blocked, no footshock was administered, and the rat was allowed to explore the dark side freely for 180 s, after which it was removed back to the home cage.

A drug (the CB_1 receptor antagonist AM251 or one of the agonists WIN55,212-2 or AM404) was microinjected into the BLA at different time points to address various phases of memory processing. Drugs were administered 20 min before conditioning(Pre-Cond), 20 min before the first extinction trial (pre-Ext1), or immediately (i.e., 2 min) after the first extinction trial (post-Ext1). The vehicle was administered at the same time points.

Elevated platform stress. An elevated platform (EP) (12 × 12 cm) stressor was used to examine the effects of exposure to a stressful experience on IA conditioning and extinction. Individual animals were placed on an elevated platform for 30 min in a brightly lit room, which elicits stress responses in the form of behavioral "freezing," that is, immobility for up to 10 min, defecation, and urination (Maroun and Akirav, 2008).

Exposure to the EP occurred immediately before conditioning (Pre-Cond), immediately before Ext1 (Pre-Ext1), or immediately after Ext1 (Post-Ext1). The EP groups (i.e., EP Pre-Cond, EP Pre-Ext1, and EP Post-Ext1) experienced the EP stressor in the absence of any microinjection, whereas the WIN+EP groups were microinjected with WIN55,212-2, 2 min before experiencing the EP stressor. The vehicle groups were microinjected with vehicle when the WIN+EP groups received WIN but did not experience the EP stressor.

Open field. The open field consisted of a closed wooden box. The walls were painted black, and the floor was white and divided by 1-cm-wide black lines into 25 squares measuring 10 × 10 cm each. A video image of the entire open field was displayed on a television monitor, and the movements of the rat, which was initially placed in a corner of the field, were manually recorded and analyzed to measure motor activity over a period of 5 min. Recordings were made of the time the rat spent in the central and the peripheral squares, the number of instances of rearing, and the total distance covered. The open-field arena was thoroughly cleaned between each trial.

Rats were microinjected with the different drugs into the BLA and, after 20 min, tested in the open-field arena. For rats that were placed on the EP for 30 min with or without previous microinjection of WIN55,212-2 into the BLA, the open-field test was performed immediately after the EP stressor.

Pain sensitivity. Pain sensitivity was assessed by determining the footshock intensity (in milliamperes) that elicited a discomfort response (i.e., flinch or vocalization) (Kim et al., 1991). Rats were individually placed in a Plexiglas box (25 × 25 × 34 cm) with a floor consisting of 13 stainless steel rods of 5 mm diameter, spaced every 1 cm. Each rat received a continuously ascending mild electric footshock (beginning at 0.0 mA and ending as soon as the animal flinched or vocalized) via the metal grid floor to determine current thresholds at which each animal would exhibit a flinch or a vocalization response. Two observers scored flinch and vocalization thresholds. Rats were taken for the pain sensitivity test 5 min after the open-field test.

Corticosterone measurement. Trunk blood was collected after decapitation between 9:00 and 11:00 A.M. for 4 consecutive days (from one-quarter of the rats per group per day). Samples were centrifuged at 3000 rpm for 20 min at 4°C. Serum was stored at −80°C and analyzed for CORT using ELISA kits (DSL Inc.).

Histology. On completion of the inhibitory avoidance experiments, the animals were deeply anesthetized with 4.8 ml/kg Equithesin (see above) and microinjected into the BLA with 0.5 μl of ink, to verify the location of the cannulae. Figure 1 shows a representative schematic drawing of the placements of the cannulae in the BLA (coronal view at position 3.14 and 3.30 mm posterior to bregma) (Paxinos and Watson, 1998).

Statistical analysis. The results are expressed as means ± SEM. For statistical analysis, repeated-measures ANOVA, one-way ANOVA, and *t* tests were used as indicated. All *post hoc* comparisons were made using the least-significant difference multiple-comparison test.

Results

Cannabinoid receptor agonist WIN55,212-2 microinjected into the BLA has no effect on inhibitory avoidance conditioning or extinction

First, we asked whether stimulation of cannabinoid receptor signaling in the BLA might accelerate the IA extinction rate or affect IA conditioning. Thus, vehicle or the CB_1/CB_2 receptor agonist WIN55,212-2 were microinjected into the BLA before conditioning, before Ext1, or immediately after Ext1.

Microinjecting vehicle into the BLA before conditioning, before Ext1, or immediately after Ext1 had no effect on the latency of the rats to enter the dark side of the box ($F_{(2,9)} < 1$; NS). Consequently, all vehicle groups for the light–dark IA experiments involving WIN55,212-2 (5 μg/0.5 μl) were pooled for all analyses (vehicle, $n = 12$). For WIN55,212-2 (5 μg/0.5 μl) microinjected before conditioning (Pre-Cond WIN_5, $n = 8$), before Ext1 (Pre-Ext1 WIN_5, $n = 9$), or immediately after Ext1 (Post-Ext1 WIN_5, $n = 9$), repeated-measures ANOVA [treatment × days (4 × 4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(3,34)} < 1$; NS) (Fig. 2*a*). Also, there were no within-subject differences in the latency between the days ($F_{(1,34)} < 1$; NS), nor was there an interaction effect ($F_{(3,34)} < 1$; NS). Because of the apparent reduction in latency in the Pre-Ext1 WIN_5 group on the first extinction day, we analyzed the latency on Ext1 using one-way ANOVA, which did not reveal a significant effect ($F_{(3,34)} = 1.43$; NS).

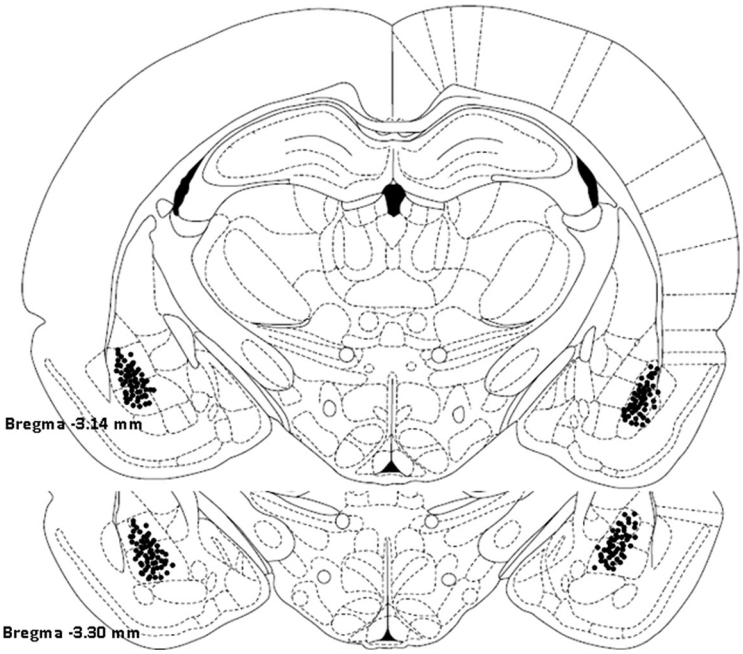

Figure 1. Representative schematic drawing of cannulae tip positions in the BLA. A coronal view at position 3.14 and 3.30 mm posterior to bregma.

Because dose–response issues may have been responsible for the failure of a microinjection of WIN55,212-2 into the BLA to affect latency, we examined the effects of other doses. Thus, the effect on latency was examined after microinjection of a lower [2.5 μg/0.5 μl (WIN_2.5), $n = 7$] or a higher [10 μg/0.5 μl (WIN_10), $n = 7$] dose of WIN55,212-2 into the BLA after Ext1. Repeated-measures ANOVA [treatment × days (3 × 4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,21)} < 1$; NS) (Fig. 2*b*). Also, there were no within-subject differences in the latency between the days ($F_{(1,21)} = 1.81$; NS), nor was there an interaction effect ($F_{(2,21)} < 1$; NS). Thus, together with the results from Figure 2*a*, WIN55,212-2 microinjected into the BLA appears to have no effect on IA conditioning or extinction by itself.

A previous report (Chhatwal et al., 2005) showed that the CB_1/CB_2 receptor agonist WIN55,212-2, and an inhibitor of endocannabinoid reuptake and breakdown, AM404, have different effects on the extinction of contextual fear. Hence, we examined the effects of AM404 on the conditioning and extinction of IA.

Microinjecting vehicle into the BLA before conditioning, before Ext1, or immediately after Ext1 had no effect on the latency of rats to enter the dark side of the box ($F_{(2,10)} < 1$; NS). Consequently, all vehicle groups in the light–dark IA experiments involving AM404 were pooled for all analyses (vehicle; $n = 13$).

For AM404 microinjected before conditioning (Pre-Cond 404, $n = 12$), before Ext1 (Pre-Ext1 404, $n = 7$), or immediately after Ext1 (Post-Ext1 404, $n = 10$), repeated-measures ANOVA [treatment × days (4 × 4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(3,38)} < 1$; NS) (Fig. 2*c*). Also, there were no within-subject differences in the latency between the days ($F_{(1,38)} < 1$; NS), nor was there an interaction effect ($F_{(3,38)} = 1.157$; NS). Because of the apparent reduction in latency in the Pre-Ext1 404 group on the first extinction day, we analyzed the latency on Ext1 using one-way ANOVA, which revealed a significant group effect ($F_{(3,38)} = 4.04$; $p = 0.014$). *Post hoc* comparison showed a significant difference between the vehicle and the Pre-Ext1 404 group ($p = 0.002$) on Ext1, indicating a reduction in the latency to enter the dark side after microinjection of AM404 that recovered the following day. Using a higher dose of AM404 (800 ng/0.5 μl) before the first extinction trial resulted in a similar effect, i.e., reduced latency to enter the dark side on Ext1 (vehicle, 118.03 ± 4.1 s, $n = 7$; Pre-Ext1 404_800, 31.74 ± 3.72 s, $n = 7$; $t_{(12)} = 5.17$; $p < 0.0001$), with no effect on Cond, Ext2, or Ext3 (data not shown). Thus, except for the transient effect on latency on Ext1, AM404 had no effect on IA conditioning or extinction.

Because the cannabinoid receptor agonist WIN55,212-2 microinjected into the BLA had no effect on IA conditioning or extinction, we next examined whether the CB_1 receptor in the BLA is essential for IA conditioning or extinction. Hence, rats were microinjected with vehicle or the CB_1 receptor antagonist AM251 before conditioning, before Ext1, or immediately after Ext1.

Microinjecting vehicle into the BLA before conditioning, before Ext1, or immediately after Ext1 had no effect on the latency of rats to enter the dark side of the box ($F_{(2,11)} < 1$; NS). Consequently, all vehicle groups for light–dark IA experiments involving AM251 were pooled for all analyses (vehicle; $n = 14$).

For AM251 microinjected rats, repeated-measures ANOVA [treatment × days (4 × 4)] revealed a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(3,38)} = 9.63$; $p < 0.001$) (Fig. 2*d*). *Post hoc* comparison unveiled a significant difference between the vehicle group and the groups microinjected with AM251 before conditioning (Pre-

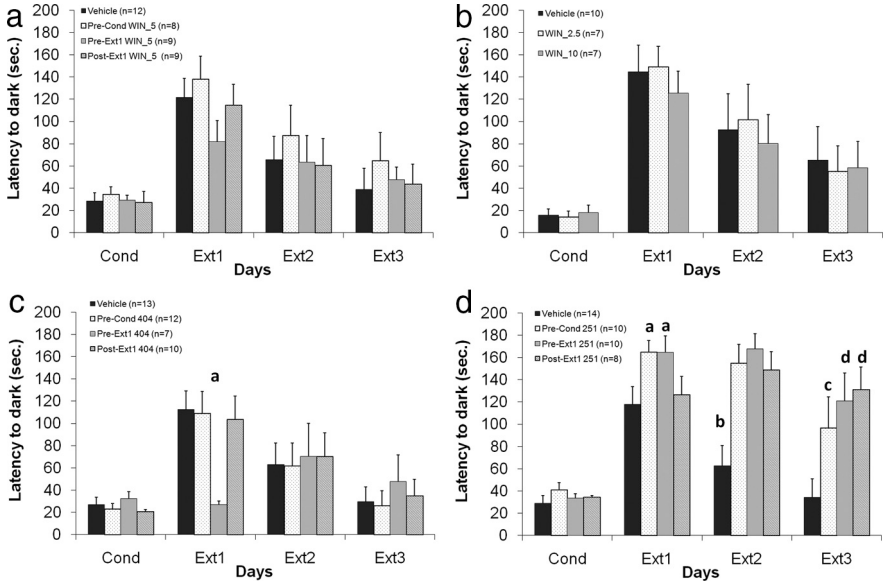

Figure 2. Cannabinoid receptor agonist WIN55,212-2 microinjected into the BLA has no effect on inhibitory avoidance conditioning or extinction. ***a***, Rats were microinjected into the BLA with vehicle ($n = 12$), with WIN55,212-2 (5 μg/0.5 μl) before conditioning (Pre-Cond WIN_5, $n = 8$), before the first extinction trial (Pre-Ext1 WIN_5, $n = 9$), or immediately after that trial (Post-Ext1 WIN_5, $n = 9$). There were no significant differences between the latencies of the groups. ***b***, Rats were microinjected into the BLA with vehicle ($n = 10$) or with a lower (2.5 μg/0.5 μl; WIN_2.5, $n = 7$) or a higher (10 μg/0.5 μl; WIN_10, $n = 7$) dose of WIN55,212-2 immediately after Ext1. There were no significant differences between the latencies of the groups. ***c***, Rats were microinjected into the BLA with vehicle ($n = 13$) or with AM404 (200 ng/0.5 μl) before conditioning (Pre-Cond 404, $n = 12$), before the first extinction trial (Pre-Ext1 404, $n = 7$), or immediately after that trial (Post-Ext1 404, $n = 10$). The latency of the Pre-Ext1 404 group was significantly shorter than that of the vehicle group on the first extinction day (Ext1, $^{a}p < 0.01$) (for details, see Results). ***d***, Rats were microinjected into the BLA with vehicle ($n = 14$) or AM251 (6 ng/0.5 μl) before conditioning (Pre-Cond 251, $n = 10$), before the first extinction trial (Pre-Ext1 251, $n = 10$), or immediately after that trial (Post-Ext1 251, $n = 8$). The latencies of all the AM251-injected groups were significantly longer than that of the vehicle group, indicating enhancement of inhibitory avoidance acquisition and/or consolidation and impaired extinction. (Ext1, $^{a}p < 0.05$, vehicle different from Pre-Cond 251 and Pre-Ext1 groups; Ext2, $^{b}p < 0.001$, vehicle different from all the groups; Ext3, $^{c}p < 0.05$, vehicle different from Pre-Cond 251; $^{d}p < 0.01$, vehicle different from Pre-Ext1 251 and Post-Ext1 251 groups).

Cond 251, $n = 10$; $p < 0.001$), before Ext1 (Pre-Ext1 251, $n = 10$; $p < 0.001$), or after Ext1 (Post-Ext1 251, $n = 8$; $p = 0.001$).

One-way ANOVA applied on each day revealed that the significant main effect stemmed from a difference in latency between the AM251-treated groups and the vehicle group throughout the extinction days (Ext1, $F_{(3,38)} = 3.12$, $p = 0.037$; Ext2, $F_{(3,38)} = 9.44$, $p < 0.001$; Ext3, $F_{(3,38)} = 4.5$, $p = 0.008$) but not on the conditioning day. *Post hoc* comparison revealed a significant difference between the vehicle group and the Pre-Cond 251 and Pre-Ext1 251 groups ($p = 0.02$) on Ext1, and between the vehicle group and all the treatment groups on Ext2 ($p < 0.001$) and Ext3 (Pre-Cond 251, $p = 0.039$; Pre-Ext1 251, $p = 0.005$; Post-Ext1 251, $p = 0.004$).

Thus, AM251 microinjected before conditioning enhanced IA acquisition and/or consolidation, as indicated by a higher latency to enter the dark side of the box on Ext1, and impaired extinction, as indicated by a higher latency to enter the dark side on Ext2 and Ext3. When AM251 was microinjected before the first extinction trial, it enhanced IA retrieval and impaired extinction. Finally, AM251 microinjected after Ext1 impaired the consolidation of IA extinction, as shown by the increased latency on Ext2 and Ext3 (but not before microinjection on Ext1). Repeated-measures ANOVA also revealed significant within-subject differences in the latency between the days ($F_{(1,38)} = 22.09$; $p < 0.001$) and a significant interaction effect ($F_{(3,38)} = 4.92$; $p = 0.005$). Hence, the cannabinoid receptor in the BLA is crucially involved in the conditioning and extinction of IA.

Cannabinoid receptor agonist WIN55,212-2 microinjected into the BLA blocks the effects of stress on inhibitory avoidance conditioning and extinction

To examine the effects of exposure to a stressful experience on the conditioning and extinction of IA, rats were exposed to the EP stress before conditioning, before Ext1, or immediately after Ext1. To examine whether cannabinoid receptor agonist would reverse the effects of stress on IA conditioning and extinction, WIN55,212-2 was microinjected into the BLA immediately before placing the rats on the EP (WIN+EP groups).

Before conditioning, rats were microinjected with vehicle ($n = 12$), placed on the EP for 30 min (EP Pre-Cond, $n = 9$), or microinjected with WIN55,212-2 (5 μg/0.5 μl) and immediately afterward placed on the EP for 30 min (WIN_5+EP, $n = 7$). Repeated-measures ANOVA [treatment × days (3×4)] revealed a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,25)} = 4.57$; $p = 0.02$) (Fig. 3*a*). *Post hoc* comparison unveiled a significant difference between the vehicle and the EP Pre-Cond group ($p = 0.006$).

One-way ANOVA applied on the different days revealed that the significant main effect stemmed from a difference in latency between the groups on Ext1 ($F_{(2,25)} = 4.184$; $p = 0.027$) but not afterward. *Post hoc* comparison showed significantly increased latency in the EP group compared with the vehicle group ($p = 0.008$). There were no within-subject differences in the latency between the days ($F_{(1,25)} < 1$; NS), nor was there an interaction effect ($F_{(2,25)} = 1.48$; NS). Thus, exposure to the EP stressor before conditioning enhanced IA acquisition and/or consolidation on Ext1, and microinjecting WIN55,212-2 into the BLA before exposure to the EP reversed the effects of the stressor on IA conditioning, because no significant differences were observed between the vehicle and WIN_5+EP group throughout the days of the experiment.

The experiment was then repeated on another set of rats with stress exposure and drug administration placed before the first extinction day. Before Ext1, rats were microinjected with vehicle ($n = 12$), placed on the EP for 30 min (EP Pre-Ext1, $n = 9$), or microinjected with WIN55,212-2 (5 μg/0.5 μl) and immediately afterward placed on the EP for 30 min (WIN_5+EP, $n = 10$). Repeated-measures ANOVA [treatment × days (3×4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,28)} = 1.04$; NS) (Fig. 3*b*). Also, there were no within-subject differences in the latency between the days ($F_{(1,28)} = 1$; NS), nor was there an interaction effect ($F_{(2,28)} = 1.04$; NS). However, rats that were placed on the EP avoided entering the dark side on Ext1 altogether (all rats reached the maximum latency of 180 s). Thus, using one-way ANOVA on the different days, we found a significant effect on latency on Ext1 ($F_{(2,28)} = 4.81$; $p = 0.017$). *Post hoc* comparisons revealed significantly increased latency in the EP group compared

with the vehicle ($p = 0.022$) and WIN_5+EP ($p = 0.007$) groups on the first extinction day. Thus, exposure to the EP stressor before the first extinction trial enhanced IA retrieval and microinjecting WIN55,212-2 into the BLA before exposure to the EP blocked the effects of the stressor on retrieval, because no significant differences were observed between the vehicle and WIN_5+EP groups throughout the days of the experiment.

The experiment was then repeated again on a third set of rats with stress exposure and drug administration placed after the first extinction day. After Ext1, rats were microinjected with vehicle ($n = 14$), placed on the EP for 30 min (EP Pre-Ext1, $n = 8$), or microinjected with WIN55,212-2 (5 μg/0.5 μl) and immediately afterward placed on the EP for 30 min (WIN_5+EP, $n = 8$). Repeated-measures ANOVA [treatment × days (3×4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,27)} = 1.86$; NS) (Fig. 3*c*). Also, there were no within-subject differences in latency between the days ($F_{(1,27)} < 1$; NS), nor was there an interaction effect ($F_{(2,27)} = 1.37$; NS). However, rats that were placed on the EP showed increased latency to enter the dark side of the box on Ext2, and, using one-way ANOVA on the different days, we found a significant effect on the latency on Ext2 ($F_{(2,27)} = 3.4$; $p = 0.048$). *Post hoc* comparisons revealed significantly increased latency in the EP group compared with the vehicle ($p = 0.019$) and WIN_5+EP ($p = 0.05$) groups.

Thus, exposure to the EP stressor after the first extinction trial disrupted the consolidation of extinction, and microinjecting WIN55,212-2 before exposure to the EP reversed the impairing effects of the stressor, because no significant differences were observed between the vehicle and WIN_5+EP groups on the second and third extinction days.

Next we examined whether a lower dose of WIN55,212-2 (2.5 μg/0.5 μl) microinjected into the BLA after Ext1 would also block the impairing effects of the stressor on the consolidation of IA extinction. After Ext1, rats were microinjected with vehicle ($n = 8$), placed on the EP for 30 min (EP Post-Ext1, $n = 8$), or microinjected with a lower dose of WIN55,212-2 and immediately afterward placed on the EP for 30 min (WIN_2.5+EP, $n = 8$). Repeated-measures ANOVA [treatment × days (3×4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,21)} = 1.03$; NS) (Fig. 3*d*). Also, there were no within-subject differences in latency between the days ($F_{(1,21)} = 2.7$; NS), nor was there an interaction effect ($F_{(2,21)} < 1$; NS). However, rats that were placed on the EP showed increased latency to enter the dark side of the box on Ext2 (i.e., all EP Post-Ext1 rats reached the maximum latency of 180 s). Thus, using one-way ANOVA on the different days, we found a significant effect on the latency on Ext2 ($F_{(2,21)} = 4.42$; $p = 0.027$). *Post hoc* comparisons revealed significantly increased latency in the EP group compared with the WIN_2.5+EP group ($p = 0.009$) and a marginally significant difference compared with the vehicle group ($p = 0.061$). Thus,

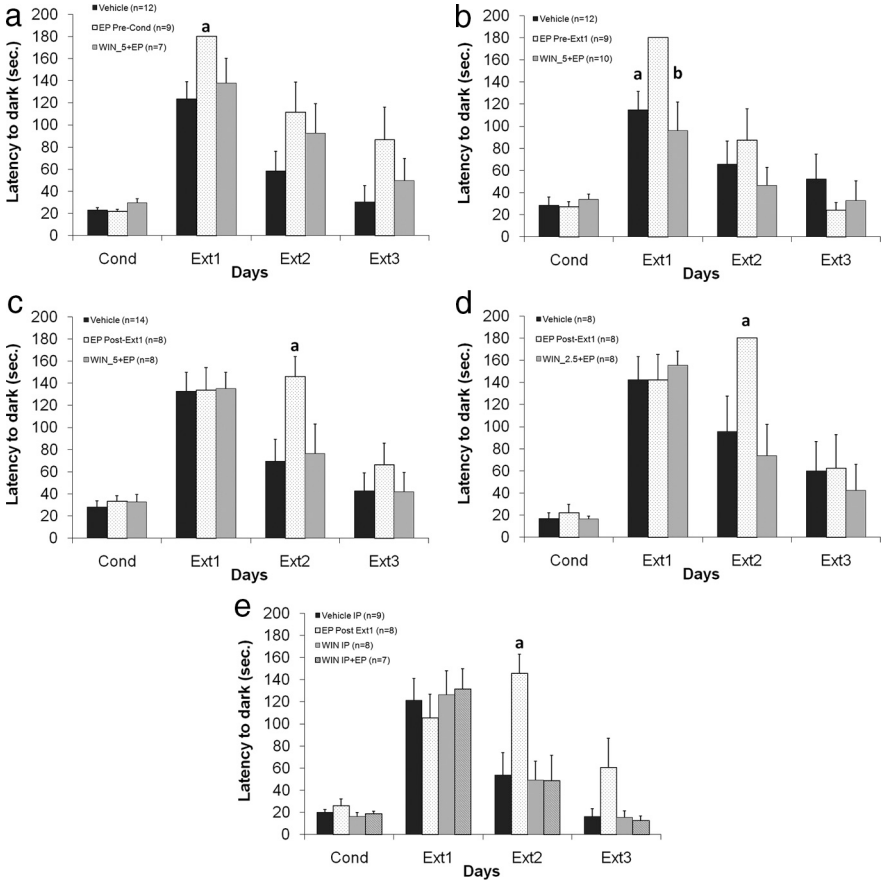

Figure 3. Cannabinoid receptor agonist WIN55,212-2 blocks the effects of EP stress on IA conditioning and extinction. ***a***, Before conditioning, rats were microinjected with vehicle ($n = 12$), placed on the EP (EP Pre-Cond, $n = 9$), or microinjected with WIN55,212-2 (5 μg/0.5 μl) and immediately afterward placed on the EP (WIN_5+EP, $n = 7$). The EP Pre-Cond group showed a significantly increased latency to enter the dark side on the first extinction day compared with the vehicle group (Ext1, $^{a}p < 0.01$). Thus, WIN55,212-2 administered into the BLA before stressor exposure reversed the enhancing effect of the stressor on IA acquisition and/or consolidation. ***b***, Before the first extinction trial, rats were microinjected with vehicle ($n = 12$), placed on the EP (EP Pre-Ext1, $n = 9$), or microinjected with WIN55,212-2 (5 μg/0.5 μl) and immediately afterward placed on the EP (WIN_5+EP, $n = 10$). The EP Pre-Ext1 group showed a significantly increased latency to enter the dark side on the first extinction day (Ext1, $^{a}p < 0.05$, EP differs from vehicle; $^{b}p < 0.01$, EP differs from WIN_5+EP). Thus, WIN55,212-2 administered into the BLA before stressor exposure reversed the enhancing effect of the stressor on IA retrieval. ***c***, After the first extinction trial, rats were microinjected with vehicle ($n = 14$), placed on the EP (EP Post-Ext1, $n = 8$), or microinjected with WIN55,212-2 (5 μg/0.5 μl) and immediately afterward placed on the EP (WIN_5+EP, $n = 8$). The EP Post-Ext1 group showed a significantly increased latency to enter the dark side on the second extinction day compared with the other groups (Ext2, $^{a}p < 0.05$). Thus, WIN55,212-2 administered into the BLA before stressor exposure reversed the disrupting effect of the stressor on IA extinction. ***d***, After the first extinction trial, rats were microinjected with vehicle ($n = 8$), placed on the EP (EP Post-Ext1, $n = 8$), or microinjected with a low dose of WIN55,212-2 (2.5 μg/0.5 μl) and immediately afterward placed on the EP (WIN_2.5+EP, $n = 8$). The EP Post-Ext1 group showed a significantly increased latency to enter the dark side on the second extinction day (Ext2, $^{a}p < 0.01$, EP Post-Ext1 differs from WIN_2.5+EP). Thus, a lower dose of WIN55,212-2 administered into the BLA before stressor exposure also reversed the disrupting effect of the stressor on IA extinction. ***e***, After the first extinction trial, rats were intraperitoneally injected with vehicle ($n = 9$), placed on the EP (EP Post-Ext1, $n = 8$), intraperitoneally injected with WIN (0.25 mg/kg; WIN IP, $n = 8$), or intraperitoneally injected with WIN and immediately afterward placed on the EP (WIN IP+EP, $n = 7$). The EP Post-Ext1 group showed a significantly increased latency to enter the dark side on the second extinction day compared with all the other groups (Ext2, $^{a}p < 0.01$). Thus, intraperitoneal administration of WIN55,212-2 before stressor exposure also reversed the disrupting effect of the stressor on IA extinction.

Table 1. The effects of cannabinoid receptor agonists and antagonist microinjected into the BLA on locomotion and anxiety in the open-field test

	Vehicle ($n = 6$)	AM404 ($n = 6$)	WIN55,212-2 ($n = 6$)	AM251 ($n = 6$)
Time in center (s)	7.83 ± 1.25	6.33 ± 1.31	4.66 ± 1.08	4.5 ± 1.28
Time in periphery (s)	292.16 ± 1.25	293.66 ± 1.31	295.33 ± 1.08	295.5 ± 1.28
Number of rearing events	20.33 ± 1.74	21.66 ± 2.03	22 ± 1.69	19.16 ± 2.10
Distance covered (s)	1758.33 ± 114.32	1916.66 ± 158.46	1675 ± 107.04	1729.16 ± 231.16

Rats microinjected into the BLA with the CB_1 receptor antagonist (AM251, $n = 6$), one of the agonists (WIN55,212-2 or AM404, $n = 6$ each), or vehicle ($n = 6$) showed no differences in any of the parameters measured in the open-field test.

microinjecting a lower dose of WIN55,212-2 into the BLA before exposure to the EP also reversed the impairing effects of the stressor on the consolidation of extinction.

Finally, we were interested in investigating whether the same effects would be seen after systemic treatment with WIN55,212-2 (0.25 mg/kg, i.p.). Hence, immediately after Ext1, rats were intraperitoneally injected with vehicle (Vehicle IP, $n = 9$), placed on the EP for 30 min (EP Post-Ext1, $n = 8$), intraperitoneally injected with WIN55,212-2 (WIN IP, $n = 8$), or intraperitoneally injected with WIN55,212-2 and immediately afterward placed on the EP for 30 min (WIN IP+EP, $n = 7$). Repeated-measures ANOVA [treatment × days (3×4)] revealed a strong trend in terms of the latency to enter the dark side of the box ($F_{(3,28)} = 2.61$; $p = 0.07$) (Fig. 3*e*). One-way ANOVA applied on the different days revealed a significant difference in latency between the groups on Ext2 ($F_{(3,28)} = 5.94$; $p = 0.003$). *Post hoc* comparison showed significantly increased latency in the EP group compared with the other groups ($p = 0.002$). Thus, systemic administration of WIN55,212-2 before exposure to the EP also reversed the impairing effects of the stressor on the consolidation of extinction. Repeated-measures ANOVA also revealed a significant interaction effect ($F_{(3,28)} = 5.68$; $p = 0.004$) but no within-subject differences in latency between the days ($F_{(1,28)} = 1.4$; NS).

The effects of the different manipulations on anxiety and sensorimotor parameters

Next, we performed two types of control experiments (the open-field and pain sensitivity tests) to exclude the possibility that the effects of the drugs on IA acquisition, consolidation, or extinction were caused by sensorimotor deficits or by increased anxiety under the experimental conditions used. Hence, rats were microinjected into the BLA with the CB_1 receptor antagonist (AM251, $n = 6$; 6 ng/0.5 μl), agonists [WIN_5, $n = 6$ (5 μg/0.5 μl) and AM404, $n = 6$ (200 ng/0.5 μl)], or vehicle ($n = 6$) and then tested in the open-field arena and in the pain sensitivity test. One-way ANOVA did not reveal a significant difference in any of the parameters measured in the open-field test (Table 1), namely, time spent in the center ($F_{(3,20)} = 1.65$; NS), time spent in the periphery ($F_{(3,20)} = 2.8$; NS), number of rearing events ($F_{(3,20)} < 1$; NS), or the distance covered ($F_{(3,20)} = 2.44$; NS). Also, ANOVA did not reveal significant differences in pain sensitivity ($F_{(3,20)} < 1$; NS) (Table 2).

Although WIN55,212-2 microinjected into the BLA had no effect on locomotion, anxiety, or pain sensitivity by itself, the combination of WIN55,212-2 and the EP could conceivably have a different effect on those parameters than either component alone. Hence, experiments were undertaken in which the rats were microinjected into the BLA with vehicle ($n = 6$), placed on the EP ($n = 5$), or microinjected with WIN55,212-2 and placed on the EP (WIN_5+EP, $n = 6$) and then tested in the open-field arena and in the pain sensitivity test. In the open field, one-way ANOVA did not reveal a significant difference between the groups in terms of time spent in the center ($F_{(2,14)} < 1$; NS), time spent in the periphery ($F_{(2,14)} < 1$; NS), or the distance covered ($F_{(2,14)} = 2.17$; NS) (Table 3). However, a significant difference was found between the groups in terms of the number of rearing events ($F_{(2,14)} = 7.74$; $p = 0.005$). *Post hoc* comparisons revealed that the vehicle group reared significantly more times than the EP ($p = 0.002$) and the WIN_5+EP ($p = 0.013$) groups. Rearing behavior characterizes individual differences in reactivity to novelty, and, thus, more frequent rearing may indicate greater novelty seeking behavior (i.e., less anxiety) (Thiel et al., 1999). The EP group showed a reduced number of rearing events and a trend toward a reduced distance covered in the open-field test compared with the control group, thus suggesting an increased stress level that may have contributed to the enhanced IA acquisition or consolidation and disrupted extinction shown in the previous figures.

Finally, one-way ANOVA did not reveal significant differences in pain sensitivity ($F_{(2,14)} < 1$; NS) (Table 4).

Table 2. The effects of cannabinoid receptor agonists and antagonist microinjected into the BLA on pain sensitivity

	Vehicle ($n = 6$)	AM404 ($n = 6$)	WIN55,212-2 ($n = 6$)	AM251 ($n = 6$)
Pain threshold for foot shock (mA)	0.36 ± 0.04	0.31 ± 0.03	0.30 ± 0.01	0.34 ± 0.03

Rats microinjected into the BLA with the CB_1 receptor antagonist (AM251, $n = 6$), one of the agonists (WIN55,212-2 or AM404, $n = 6$ each), or vehicle ($n = 6$) showed similar pain sensitivity responses to electric footshock.

Table 3. The effects of WIN 55,212-2 and the EP on locomotion and anxiety in the open-field test

	Vehicle ($n = 6$)	EP ($n = 5$)	WIN55,212-2 + EP ($n = 6$)
Time in center (s)	9.5 ± 0.76	7.8 ± 4.18	5.5 ± 1.91
Time in periphery (s)	290.5 ± 0.76	292.2 ± 4.18	294.5 ± 1.91
Number of rearing events	19.16 ± 1.25	10.4 ± 2.28*	12.83 ± 1.1**
Distance covered (s)	1525 ± 163.17	1080 ± 180.62	1258.33 ± 84.07

Rats placed on the EP ($n = 5$) showed increased rearing in the open-field test compared with groups that received a microinjection of vehicle ($n = 6$) or WIN55,212-2 before being placed on the platform (WIN_5+EP; $n = 6$) (*$p < 0.05$, vehicle group differs from WIN_5+EP group; **$p < 0.01$, vehicle group differs from EP group).

Table 4. The effects of WIN55,212-2 and the EP on pain sensitivity

	Vehicle ($n = 6$)	EP ($n = 5$)	EP+WIN55,212-2 ($n = 6$)
Pain threshold for foot shock (mA)	0.26 ± 0.01	0.24 ± 0.01	0.24 ± 0.01

Rats microinjected into the BLA with vehicle ($n = 6$), placed on the EP ($n = 5$), or microinjected with WIN55,212-2 and placed on the EP (WIN_5 + EP, $n = 6$) showed similar pain sensitivity responses to electric footshock.

WIN55,212-2 microinjected into the BLA or administered intraperitoneally reduces stress-induced increases in corticosterone levels

Because it has been suggested that the augmentation of endocannabinoid signaling can suppress stress-responsive systems

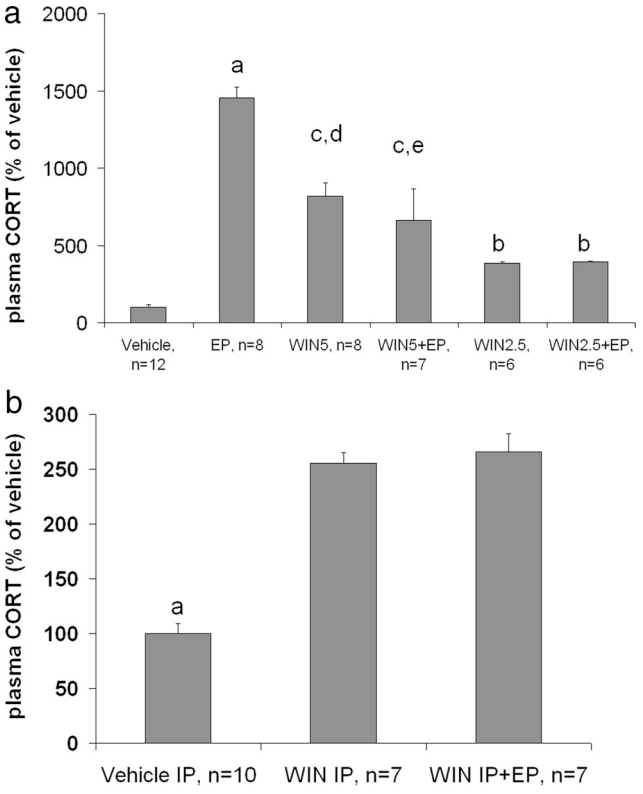

Figure 4. The effects of the cannabinoid receptor agonist WIN55,212-2 and EP stress on CORT levels. ***a***, CORT levels were measured in rats microinjected with vehicle into the BLA (vehicle, $n = 12$), placed on the EP ($n = 8$), microinjected with WIN55,212-2 (5 μg/0.5 μl) into the BLA (WIN_5, $n = 8$), microinjected with WIN55,212-2 (5 μg/0.5 μl) into the BLA and placed on the EP (WIN_5+EP, $n = 7$), microinjected with a lower dose of WIN55,212-2 (2.5 μg/0.5 μl) into the BLA (WIN_2.5, $n = 6$), or microinjected with the lower dose of WIN55,212-2 and placed on the EP (WIN_2.5+EP, $n = 6$). Data represent the means ± SEM expressed as a percentage of the CORT values of the vehicle animals (CORT levels in the vehicle group, 95.52 ± 16.7 ng/ml) ($^{a}p < 0.001$, EP group differs from all other groups; $^{b}p < 0.05$ and $^{c}p < 0.001$, vehicle group differs from all other groups; $^{d}p < 0.01$, WIN_5 group differs from WIN_2.5 and WIN_2.5+EP groups; $^{e}p < 0.05$, WIN_5+EP group differs from WIN_2.5 and WIN_2.5+EP groups). ***b***, CORT levels were measured in rats injected intraperitoneally with vehicle (Vehicle IP, $n = 10$), WIN55,212-2 (WIN IP, $n = 7$), or injected with WIN55,212-2 intraperitoneally and placed on the EP (WIN IP+EP, $n = 7$). Data represent the means ± SEM expressed as a percentage of the CORT values of the vehicle animals (CORT levels in the vehicle group, 381.01 ± 64.39 ng/ml) ($^{a}p < 0.001$, vehicle group differs from all other groups).

(Patel et al., 2004; Cota, 2008; Steiner and Wotjak, 2008), we sought to examine whether WIN55,212-2 given in conjunction with EP had a different effect on CORT levels than did exposure to the stressor alone.

In the first CORT experiment, rats were microinjected with vehicle to the BLA (vehicle, $n = 12$), placed on the EP ($n = 8$), microinjected with WIN55,212-2 (5 μg/0.5 μl) into the BLA (WIN_5, $n = 8$), microinjected with WIN55,212-2 (5 μg/0.5 μl) and placed on the EP (WIN_5 +EP, $n = 7$), microinjected with a lower dose of WIN55,212-2 (2.5 μg/0.5 μl) into the BLA (WIN_2.5, $n = 6$), or microinjected with the lower dose of WIN55,212-2 and placed on the EP (WIN_2.5+EP, $n = 6$).

Thirty minutes after microinjection (vehicle and WIN groups) or immediately after the EP (EP and WIN+EP groups), trunk blood was collected for CORT measurement. One-way ANOVA on CORT levels unveiled a significant difference between the groups ($F_{(5,41)} = 32.7$; $p < 0.001$) (Fig. 4*a*). *Post hoc* comparisons revealed that rats that were exposed to the EP in the absence of previous WIN microinjection, i.e., the EP group, showed the highest CORT levels when compared with all the groups ($p < 0.001$). The vehicle group showed the lowest CORT levels and was significantly different from all the groups (WIN_5 and WIN_5+EP, $p < 0.001$; WIN_2.5 and WIN_2.5+EP, $p < 0.05$). Also, the WIN_2.5 and WIN_2.5+EP groups showed significantly lower CORT levels than the WIN_5 ($p < 0.01$) and WIN_5+EP groups ($p < 0.05$). Hence, WIN55,212-2 microinjection into the BLA (2.5 μg/0.5 μl or 5 μg/0.5 μl) in itself increased CORT levels compared with those of the vehicle group, but it reduced CORT levels in rats that were exposed to the EP stress when compared with rats exposed to the EP without WIN microinjection. Furthermore, although both WIN doses reversed the stress-induced increase in CORT levels, the effect was dose dependent, because a lower dose of WIN resulted in less CORT activation than did the higher dose of WIN.

In the second CORT experiment, rats were injected intraperitoneally with vehicle (Vehicle IP, $n = 10$) or WIN55,212-2 (WIN IP, $n = 7$), or injected with WIN55,212-2 and placed on the EP (WIN IP+EP, $n = 7$).

Thirty minutes after injection (vehicle and WIN groups) or immediately after the EP (WIN+EP group), trunk blood was collected for CORT measurement. It seems that the injection of the vehicle intraperitoneally is stressful by itself because the intraperitoneal vehicle group showed relatively enhanced CORT levels (CORT levels in the vehicle group, 381.01 ± 64.39 ng/ml). Nevertheless, one-way ANOVA on CORT levels unveiled a significant difference between the groups ($F_{(2,21)} = 39.11$; $p < 0.001$) (Fig. 4*b*). *Post hoc* comparisons revealed that the vehicle rats showed significantly lower CORT levels than the WIN IP and WIN IP+EP groups ($p < 0.001$). Hence, WIN55,212-2 injected intraperitoneally in itself increased CORT levels compared with those of the vehicle group, but it reduced CORT levels in rats that were exposed to the EP stress when compared with rats exposed to the EP without WIN injection (EP) (shown in Fig. 4*a*).

Finally, we examined whether the effects of AM251 microinjected into the BLA on IA conditioning and extinction are associated with alterations in CORT levels. *t* test unveiled a significant increase in CORT levels in rats microinjected with AM251 into the BLA [AM251 BLA, $n = 7$; plasma CORT levels (% of vehicle), 199.8 ± 40.8 ng/ml] compared with the vehicle group (Vehicle BLA, $n = 10$; CORT levels, 100 ± 25.72 ng/ml) ($t_{(15)} = 2.16$; $p = 0.047$).

Discussion

The main finding of the present study is that cannabinoid receptor activation in the BLA reverses the enhancing effects of environmental stress on IA conditioning and its impairing effects on extinction. We also find that WIN55,212-2 microinjected into the BLA inhibits stress-induced corticosterone elevation, thus suggesting that the reversal of the effects of stress on memory caused by cannabinoid activation in the BLA may be associated with influences on the HPA axis. Furthermore, the results show the crucial involvement of the CB_1 receptor in the BLA in the extinction of avoidance behavior because the CB_1 receptor antagonist impairs IA extinction. The control experiments demonstrate that the effects of WIN55,212-2 cannot be attributed to sensorimotor deficits, because these parameters seemed unchanged by WIN55,212-2 microinjected into the BLA. Together, these findings suggest that the BLA could be an important neural substrate relevant to the effects of cannabi-

noids on emotional responses and that cannabinoids may have a potential therapeutic value in the treatment of fear- and stress-related disorders.

The effects of CB_1 receptor antagonist AM251 on inhibitory avoidance learning

Administration of the CB_1 receptor antagonist into the BLA before conditioning or before/after the first extinction trial potentiates the aversive response or blocks extinction of IA. Indeed, the importance of CB_1 receptors in the extinction of aversive memories has been substantiated by several groups in different behavioral paradigms using systemic administration. CB_1 receptor antagonists were found to impair extinction in fear-related (Marsicano et al., 2002; Suzuki et al., 2004; Chhatwal et al., 2005; Reich et al., 2008) and non-fear-related paradigms (Varvel and Lichtman, 2002), with no effect on appetitively motivated learning tasks (Hölter et al., 2005; Niyuhire et al., 2007; Harloe et al., 2008). Reich et al. (2008) found that administrating AM251 enhances acquisition of freezing behavior and impairs extinction in trace and delay pavlovian fear conditioning. However, several studies did not find the CB_1 receptor antagonist to have any effect on memory acquisition or consolidation (Marsicano et al., 2002; Suzuki et al., 2004; De Oliveira Alvares et al., 2008). Recently, it has been suggested that the endocannabinoid system prevents the expression of inappropriate generalized and learned responses during aversive learning and retention (Reich et al., 2008), thus, possibly explaining the enhancing effects of the CB_1 receptor antagonist on IA learning and its impairing effects on extinction.

Memory retrieval is thought to activate a second memory consolidation cascade (i.e., reconsolidation) or it may initiate the opposite process of extinction (Nader et al., 2000; Sara, 2000; Dudai, 2002; Alberini, 2005). Reconsolidation acts to stabilize, whereas extinction tends to weaken, the expression of the original memory. It has been suggested that, after retrieval, there is a brief time window for reconsolidation, whereas extinction only occurs after prolonged reexposure, and that the process that prevails is determined (at least partly) by the duration of the reexposure (Suzuki et al., 2004). Here, the latencies of the control rats to enter the dark side decreased over repeated tests, thus supporting the extinction process. Accordingly, we suggest that AM251 microinjected into the BLA impairs IA extinction rather than facilitates reconsolidation.

The effects of cannabinoid receptor agonists WIN55,212-2 and AM404 on inhibitory avoidance learning

WIN55,212-2, in doses ranging from 2.5 to 10 μg/0.5 μl, administered into the BLA has no effect on IA conditioning or on extinction kinetics. AM404 microinjected before the first extinction trial reduces the latency to enter the dark side on Ext1, with latency recovering the following day. Thus, the drug may elicit a general decrease in the inhibitory response that temporarily affects the rats' latency. Chhatwal et al. (2005) have shown that AM404 facilitates the retention of extinction of conditioned fear, whereas WIN55,212-2 has no effect. However, Pamplona et al. (2006) found that WIN55,212-2 facilitates the extinction of both contextual fear memory and a reversal task in the water maze. Using intracerebral injection, Kobilo et al. (2007) found that WIN55,212-2 has no effect on the extinction of conditioned taste aversion. Thus, the alleviating effects of cannabinoid receptor activation on extinction have not been observed consistently.

Many studies have shown that the administration of CB_1 receptor agonists impairs memory (Lichtman et al., 1995; Hampson and Deadwyler, 1999; Davies et al., 2002). However, several other studies have indicated differently, in particular with regards to aversive or fear-based paradigms. For example, CB_1 receptor agonist enhances the acquisition of contextual fear conditioning (Mikics et al., 2006) but has no effect on the acquisition of other aversive tasks (De Oliveira Alvares et al., 2008; Yim et al., 2008). Thus, cannabinoids may have various effects that may result from differences in experimental protocols (e.g., aversive vs nonaversive protocols, mass vs spaced extinction trials, time of drug injection or time between extinction learning and testing, central or systemic drug administration, the use of different drugs, etc).

Cannabinoid receptor agonist in the BLA reverses the effects of stress on inhibitory avoidance learning

Exposing rats to acute stress before conditioning or before/after the first extinction trial enhances inhibitory acquisition/consolidation and disrupts extinction. This corroborates several studies that examined the effects of stress on different memory processes (Cordero et al., 2003; Izquierdo et al., 2006; Akirav and Maroun, 2007). Although administering the cannabinoid receptor agonist into the BLA has no effect on IA conditioning and extinction by itself, environmental stress and cannabinoid receptor activity interact in their regulation of memory in the BLA. Thus, cannabinoid activation in the BLA acts to modulate the effects of stress on conditioning and extinction. In support, Patel et al. (2005) found a synergistic interaction between environmental stress and CB_1 receptor activation in the amygdala, because the combination of restraint stress and CB_1 agonist administration produces robust Fos induction within the BLA and the central amygdala.

The effects of cannabinoids and stress on corticosterone levels

Intra-BLA WIN55,212-2 by itself dose dependently enhances CORT levels when compared with the control group, because the higher dose (5 μg/0.5 μl) resulted in more CORT secretion than the lower dose (2.5 μg/0.5 μl). This is consistent with findings that cannabinoid activation in both human and animal models stimulates glucocorticoid secretion (Murphy et al., 1998). Most importantly, the CORT levels of rats microinjected with WIN55,212-2 into the BLA without exposure to the EP stressor do not differ significantly from those of rats microinjected with WIN55,212-2 and then exposed to the stressor. Similarly we found that an intraperitoneal administration of WIN55,212-2 (0.25 mg/kg) reversed the stress-induced increase in CORT levels. Hence, acute stress elevates corticosterone levels, and CB_1 receptor activation in the BLA significantly reduces this stress-induced elevation. These findings may suggest that cannabinoid activation in the BLA modulates the effects of stress on learning, at least partially, via inhibition of the HPA axis. Similarly, Patel et al. (2004) have demonstrated that mice treated systemically with CB_1 receptor agonists show significantly decreased or eliminated restraint-induced CORT release. In our study, the abolishment of the effects of stress on CORT levels by WIN55,212-2 was localized to the BLA. Interestingly, microinjecting the CB_1 receptor antagonist AM251 (6 ng/0.5 μl) also resulted in the enhancement of CORT levels.

A model that explains the possible interaction between the endocannabinoid system, stress and the HPA axis has been

suggested previously (Patel et al., 2005; Cota, 2008). On exposure to an acute stressor, a reduction in endocannabinoid signaling would result in increased synaptic activity at glutamatergic afferents to the paraventricular nucleus (PVN), thus allowing stressful stimuli to activate the HPA axis (Di et al., 2003; Patel et al., 2004). The BLA has received considerable attention as a stress-regulatory structure, but there is limited evidence of direct innervations of the PVN by the BLA or other intra-amygdalar projections of the BLA, such as the medial and central nuclei (Herman et al., 2003). Hence, the mechanism by which WIN55,212-2 administered into the BLA inhibits the HPA axis during stress needs additional investigation. In any case, it is important to note that pharmacological administration of exogenous cannabinoids may lead to a different action than that induced by the endogenous agents of the endocannabinoid system. Thus, exogenous CB_1 receptor activation, as in our study, may not resemble endocannabinoid signaling and its role in HPA axis regulation (Steiner and Wotjak, 2008).

It has been shown recently (Campolongo et al., 2009) that the endocannabinoid system is involved in modulating the consolidation of memory for IA training and that CB_1 activity within the BLA is essential for mediating glucocorticoid effects on long-term IA memory. Specifically it has been shown that AM251 administered into the BLA prevented CORT effects on memory consolidation. Steiner et al. (2008) have shown that mice lacking CB_1 in cortical glutamatergic neurons showed decreased immobility in the forced swim test with normal corticosterone release compared with controls. In our study, AM251 into the BLA was found to facilitate and impair IA conditioning and extinction, respectively, and to increase CORT levels. Exposure to the EP stress had similar effects on both IA learning and CORT levels. Together, it seems that additional investigation regarding the possible interaction between the CB_1 receptor antagonist and the HPA axis is required.

The modulation of emotional processes by cannabinoids

Cannabis is widely used, primarily because of its euphorant, anti-anxiety, and stress-reducing properties (Green et al., 2003). The effects of cannabinoid agonists on anxiety are biphasic, with low doses being anxiolytic and high doses anxiogenic (Viveros et al., 2005). Although the precise mechanisms by which CB_1 receptors modulate neuronal activity within the BLA are not fully understood, various studies have reported that cannabinoids serve to attenuate the neuronal and behavioral responses to aversive environmental stimuli (Patel et al., 2005). Indeed, pharmacological augmentation of cannabinoids reduces anxiety-related behavioral responses (Berrendero and Maldonado, 2002; Kathuria et al., 2003) and suppresses restraint stress-induced corticosterone release (Patel et al., 2004). In addition, cannabinoid exposure was shown to decrease corticotropin-releasing hormone levels in the amygdala, which may account for reduced stress responses (Rodríguez de Fonseca et al., 1997).

Within the BLA, high concentrations of CB_1 receptors are found localized on a subpopulation of inhibitory interneurons (McDonald and Mascagni, 2001), suggesting an important regulatory role for CB_1 receptor transmission within the BLA through endocannabinoid signaling. Several studies have reported strong inhibition of BLA interneurons after application of CB_1 receptor agonists (Azad et al., 2004; Pistis et al., 2004), which is expected to decrease local inhibitory feedback on pyramidal amygdalar outputs neurons. Katona et al. (2001) suggested that, by reducing the tonic GABAergic inhibitory control over pyramidal cells in the BLA, cannabinoids indirectly inhibit neuronal activity in the central nucleus, which mediates stress and fear responses to aversive stimuli. Nevertheless, cannabinoids were found to control synaptic transmission in the lateral amygdala by also modulating glutamatergic synapses (Azad et al., 2003). Thus, this suggests that the effects could also result from CB_1-mediated suppression of excitatory neurotransmission.

It has been suggested that the endocannabinoid system has a specific involvement in the habituation component of fear extinction (Kamprath et al., 2006) and that this involvement resembles its role in adaptation of stress responses (Viveros et al., 2005). Patel et al. (2005) showed that the endocannabinoid system mediates habituation to repeated restraint stress and suggested that pharmacological augmentation of endocannabinoid signaling is a good target for the treatment of affective disorders (Patel and Hillard, 2008). Altogether, these studies indicate that extinction of aversive memories via a habituation-like process and the adaptation to stress responses via the alleviation of the stress axis are, in part, controlled by endocannabinoids (for review, see Lutz, 2007).

Conclusions

Our findings give preclinical support to the suggestion that cannabinoids could represent a therapeutic target for the treatment of diseases associated with the inappropriate retention of aversive memories, such as posttraumatic stress disorder (Marsicano et al., 2002). Importantly, because of the effects of the drug on the stress response, it is likely that potential patients treated with cannabinoids or related compounds might benefit also from the stress-reversing effects of the drug. Nevertheless, studies show that cannabinoids elicit dose-dependent, biphasic effects on emotionality (Onaivi et al., 1990; Haller et al., 2004; Viveros et al., 2007; Moreira et al., 2009). Thus, the dose together with the context in which cannabinoids are administered should be taken into consideration.

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