

A Request to List Post-Traumatic Stress Disorder (PTSD) as a Debilitating Condition under the Arizona Medical Marijuana Act (AMMA)

Submitted to the *Arizona Department of Health Services*

RE: Medical Advisory Committee Recommendations to the Agency Director

TABLE OF CONTENTS

2	Introduction
5	A description of the symptoms and other physiological effects experienced by an individual suffering from PTSD or a treatment of PTSD that may impair the ability of the individual to accomplish activities of daily living;
8	The availability of conventional medical treatments to provide therapeutic or palliative benefits for PTSD or a treatment of the PTSD;
10	A summary of the evidence that the use of marijuana will provide therapeutic or palliative benefits for PTSD or a treatment of PTSD; and
Exhibits	Articles published in peer-reviewed, scientific journals reporting the results of research concerning marijuana's effects on or treatment of PTSD and supporting PTSD's addition as a debilitating condition under the AMMA.

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A Request to List PTSD as a Qualifying Condition under the AMMA

In accordance with the Arizona Medical Marijuana Act (the “AMMA”), the Arizona Department of Health Services (“ADHS”) considers adding new, debilitating conditions twice a year. As noted in a letter sent by the Medical Advisory Committee on July 17, 2012, “by setting expectations for clinical assessments by medical providers and using evidence-based research to guide programmatic decisions,” ADHS maintains the medical focus of the AMMA. ADHS uses various procedures and protocols to avail itself of the full weight of scientific and other evidence presented. With recommendations provided by the Medical Committee, and under the guidance of ADHS director Will Humble, ADHS has continued to uphold the high standards of the AMMA. With this in mind, we are pleased to submit our petition for adding PTSD as a qualifying condition to the AMMA.

The issue of whether the AMMA should list PTSD as a qualifying condition balances PTSD sufferers’ freedom to choose their treatment and the amount of research on marijuana’s efficacy to treat PTSD. While, for reasons discussed below, there are not many scientific studies directly relating to this issue, this comment will present the most relevant research to date. The University of Haifa Studies—along with studies by Dr. Fraser, Dr. Marsicano, and Dr. Sisley—all provide sufficient support for cannabis’s ability to effectively treat the underlying causes of PTSD’s host of horrid symptoms.

Across the country, states are recognizing the ability of medical marijuana to better the quality of life of those suffering from PTSD. Recently, Oregon approved PTSD as a qualifying condition under its medical marijuana program.¹ Maine even more recently passed a legislative bill to include PTSD as a qualifying condition.² During its annual review, New Mexico decided that PTSD would remain a qualifying condition under its medical marijuana program.³ This groundswell of support arises not just at the State level, but also at the municipal level. The US Conference of Mayors unanimously passed a resolution at its 78th Meeting, held from June 21–24, 2013, recognizing that medical marijuana is the safest and most effective treatment option for many sufferers of PTSD.⁴ ADHS should consider the example set by these states and municipal officials as well as the scientific evidence presented in this petition and recommend including PTSD as a qualifying condition under the AMMA.

Introduction

During July 2012, ADHS received various petitions to include PTSD as a qualifying condition under the AMMA.⁵ ADHS held public hearings and accepted public comments regarding these proposals. Moreover, to further understand the efficacy of marijuana in

¹ <http://www.eastbayexpress.com/LegalizationNation/archives/2013/06/11/oregon-governor-approves-medical-marijuana-for-ptsd>.

² <http://bangordailynews.com/2013/06/30/news/state/ptsd-added-to-list-of-qualifying-conditions-for-medical-marijuana-treatment/>.

³ <http://www.drugpolicy.org/news/2013/05/access-medical-marijuana-patients-post-traumatic-stress-disorder-ptsd-new-mexico-protoc>.

⁴ <http://marijuanamajority.com/mayors/?mayor=true>

⁵ <http://www.azdhs.gov/medicalmarijuana/documents/debilitating/PTSD1.pdf>;
<http://www.azdhs.gov/medicalmarijuana/documents/debilitating/PTSD2.pdf>.

treating PTSD, ADHS assigned the University of Arizona's Colleges of Medicine and Public Health to compile an Evidence Review of published studies that address the benefits and harms of cannabis therapy for PTSD. The ADHS 2012 "Medical Advisory Committee Recommendations to the Agency Director" stated:

1) The Evidence Reviews provided by the University of Arizona's Colleges of Medicine and Public Health proved extremely helpful and informative to the Committee while developing the recommendations. 2) Because marijuana has not been subjected to any high quality, scientifically controlled testing for any of the petitioned conditions (including PTSD), we find no convincing evidence that marijuana provides a benefit. 3) We acknowledge there is anecdotal evidence that using marijuana has helped patients, but there is no way to exclude the possibility that the improvement is due solely to placebo. 4) There is also potential for harm to patients, if the Department were to approve marijuana use for these conditions. Patients may use marijuana to self medicate, and avoid seeking care from a trained medical professional. 5) Delaying initiation of appropriate, proven treatments and therapies could result in a worsening of their condition or misdiagnosis of a more serious condition. 6) While the public comments and testimony were extremely compelling, the Committee, in order to maintain the medical foundation of the program, utilized scientific evidence to guide the decisions (numbering added by authors).

We will examine each of these statements in turn:

1) The Colleges' 2012 135-page packet contains only 18 articles "that came closest to addressing any of the key questions."⁶ We disagree with the opinion that the packet was either "extremely helpful" or "informative" in providing evidence regarding the issue at hand. Our opinion is supported by the Colleges' research, which concedes, "No study was found that focused on the treatment effects of cannabis on those with PTSD."⁷ The extent of the foundational knowledge regarding symptoms of and conventional treatments for PTSD is summarized below in Parts I and II.

2) Marijuana has been subjected to high quality, scientifically-controlled testing, the findings of such testing are summarized in Part III. Although the Colleges of Medicine and Public Health's compilation of scientific studies discovered no research undertaken within the US and specifically excluded animal-based scientific studies, all must acknowledge that the National Institute on Drug Abuse's ("NIDA") evasive gatekeeping has created a research monopoly preventing the Colleges and ADHS from finding the on-point studies they seek.

3) The purpose of NIDA is to conduct and support biomedical and behavioral research, health services research, research training, and health information dissemination with respect to the prevention of drug abuse and the treatment of drug abusers.⁸ NIDA has publicly stated that it does "not fund research focused on potential medical benefits of marijuana."⁹ NIDA's executive director, Steven Gust, testified "that *it is not NIDA's mission to*

⁶ Doug Campos-Outcalt, Patricia Hamilton, and Cecilia Rosales, *Medical Marijuana for the Treatment of Post Traumatic Stress Disorder: An Evidence Review*, 3 (2012), available at www.azdhs.gov/medicalmarijuana/documents/debilitating/Debilitating-Conditions-PTSD.pdf.

⁷ *Id.*

⁸ 42 U.S.C.A. § 285(o).

⁹ NORML, *Federal Agency in Charge of Marijuana Research Admits to Stifling Studies on Medical Cannabis* (Jan. 28, 2010), available at <http://norml.org/news/2010/01/28/federal-agency-in->

*study medical uses of marijuana.*¹⁰ To conduct a study on the medicinal benefits of marijuana in the US, however, a researcher must first obtain NIDA's permission. Hence, we believe the standards used by ADHS in conducting Evidence Reviews are inappropriate under these circumstances. The State of Arizona is home to Dr. Sue Sisley—a leading researcher in the field—who has prepared and obtained FDA approval for a true scientific study of the issue. NIDA and the DEA, however, are blocking her approval and obstructing her scientific research. Arizonans understand that barricades such as these must be removed because they are unhelpful to the progress of science and medicine. By maintaining its current policies and standards,¹¹ ADHS precludes itself from examining reasonable, objective scientific analysis and drawing reasonable conclusions regarding marijuana's ability to treat PTSD. We urge ADHS to remove these artificial barriers and allow PTSD sufferers the right to choose an alternative form of medication by adding PTSD as a qualifying condition to the AMMA.

4) ADHS admits they are worried about PTSD sufferers using medical marijuana to self-medicate instead of seeking counseling, therapy and other treatment options from licensed medical practitioners. This concern, however, ignores the reason why countless PTSD sufferers have already turned to marijuana to self-medicate: current pharmacological treatments are ineffective and dangerously addictive. As we will explain, PTSD is only partially understood and it's treatment even less so. The unique brain circuitry and chemistry of each patient coupled with the unique circumstances surrounding the traumatic incident at the source of PTSD makes for a wide array of symptoms associated with the disorder. This complexity makes finding a one-size-fits-all treatment schedule for PTSD sufferers impossible, illogical, and unhealthy in ways. It's this inability of doctors to treat the full range of symptoms with pharmacological treatments that drives PTSD sufferers to self-medicate. Of the pharmacological and self-medication treatments available to PTSD sufferers, marijuana has the greatest potential for treating the largest cross-section of PTSD symptoms with the least serious side effects. Used in tandem with psychotherapy, marijuana has the potential to reverse the neurological effects of PTSD.

The importance of adding PTSD as a qualifying condition under the AMMA cannot be understated. Adding PTSD as a qualifying condition would bring those who are currently self-medicating out of the shadows and into the regulatory regime of the AMMA. These self-medicating sufferers would become patients who will receive counseling and therapy leading to a decrease—not an increase—in self-medication. Opening up this treatment

charge-of-marijuanaresearch-admits-stifling-studies-on-medicinal-cannabis-nida-does-not-fund-research-focused-on-the-potential-medical-benefits-of-marijuana.

¹⁰ Mary Ellen Bittner, In the Matter Lyle E. Craker Ph.D., Docket No. 05-16, ALJ 19 (DEA 2007). Dr. Sue Sisley of the University of Arizona discusses NIDA's monopoly on marijuana-related research here: http://www.maps.org/media/view/dr_sue_sisley_talks_about_medical_marijuana_ptsd_and_scientific_freedom/ at 2:30–3:44. Dr. Sisley helped create a political action committee called Americans for Scientific Freedom to combat this monopoly. *Id.* at 3:30–3:55.

¹¹ The College of Public Health "[e]xcluded articles includ[ing] those that were: animal studies, or experiments on biochemical or pathophysiological pathways; case reports or case series; editorials or opinions; not addressing a key question." *Supra* note 1, at 4. Again, the College of Public Health found zero studies directly testing whether marijuana can effectively treat PTSD. The Colleges' prohibitions prevent the inclusion of the only scientifically-controlled studies *directly testing the efficacy of marijuana*.

option to all sufferers could also reduce the number of PTSD sufferers who are turning to alcohol and hard drugs to self-medicate. Thus, adding PTSD as a qualifying condition under the AMMA has the potential to alleviate the very fears upon which ADHS based its rejection in 2012.

5) ADHS concedes that delay in implementing appropriate treatments could lead to a worsening of conditions for patients. The studies presented below argue for marijuana's efficacy at treating PTSD. Any delay in accessing effective treatment could lead to a worsening of conditions. A vote to list PTSD as a debilitating condition under the AMMA will help mitigate the effects of previous delays for sufferers of PTSD.

6) ADHS described the public comments of 2012 as compelling. Compelling comments from members of the public should guide their representatives on how to properly serve them. Sadly, in 2012, these compelling public comments were considered insufficient standing alone. This 2013 comment hopes to provide the additional research ADHS seeks.

I. PTSD Presents an Array of Debilitating Symptoms Affecting Everyday Life.

To be diagnosed with PTSD, an individual must be exposed to a psychologically traumatic event that facilitates the onset of persistent symptoms. These symptoms must cause significant distress or impact everyday functioning. The *Diagnostic and Statistic Manual of Mental Disorders, Fourth edition* defines a "traumatic event" as one in which "(i) the person experiences, witnesses, or was confronted with an event or events that involved actual or threatened death or serious injury or a threat to the physical integrity of self or others; and (ii) the person's response involved intense fear, helplessness, or horror." PTSD has been conceptualized as a disorder of fear in which the individual has exaggerated fear responses or the inability to control fear responses. It has also been described as a disorder of memory, in which individuals suffering from PTSD seem to "relive their trauma in the form of involuntary recollection."¹²

A. Physical Symptoms.

Physical symptoms are typically characterized by phenomena that can be grouped for the most part into three primary domains: (i) reminders of the exposure (including flashbacks, intrusive thought, nightmares); (ii) activation (including hyperarousal, insomnia, agitation, irritability, impulsivity and anger); and (iii) deactivation (including numbing, avoidance, withdrawal, confusion, derealization, dissociation, and depression).¹³ In addition to demonstrating enhanced recall for traumatic memories, distressing recollections for those with PTSD are often "vivid" and "long-lasting." It is in part these "reliving" experiences that take the form of nightmares, intrusive thoughts, and/or flashbacks—coupled with observed cognitive disturbances—that have fostered interest regarding the neurobiological and neuropsychological underpinnings of this condition.¹⁴ PTSD is characterized by the presence of signs and symptoms in the three primary domains

¹² Brenner, Lisa A., *Neuropsychological and Neuroimaging Findings in Traumatic Brain Injury and Post-traumatic Stress Disorder*.

¹³ Sherin, et al., *Post-traumatic Stress Disorder: the Neurobiological Impact of Psychological Trauma*

¹⁴ See *Supra* note 6.

described for a period extending beyond one month (such periods can in some cases occur long after the original, precipitating traumatic exposure). The signs and symptoms of PTSD, therefore, appear to reflect a persistent, abnormal adaptation of neurobiological systems to the stress of witnessed trauma.¹⁵

B. Neurological Symptoms.

Despite knowledge that genetic variability, gender, and developmental history appear to impact neurobiological systems and responses to traumatic stimuli, PTSD symptoms are believed to relate to an individual's deregulated biological response to stress. During traumatically stressful situations, neurotransmitter systems and neuroendocrine axes are activated. Neurobiological activation is thought to impact brain functioning and hypothesized to alter the structure of brain regions including the amygdala, hippocampus, locus coeruleus, dorsal raphe nucleus, and prefrontal cortex. Recent work by Eckart and colleagues noted reduced volume in the prefrontal and parietal regions of refugees with PTSD and suggested that such disturbances, along with previously reported findings regarding the medial temporal region, may highlight memory "disturbance" associated with PTSD. Among those with PTSD, findings demonstrated an exaggerated amygdala response, deficient prefrontal functioning, and decreased hippocampal activation.¹⁶

Characteristic changes in brain structure and function have been identified in patients with PTSD using brain-imaging methods. Brain regions that are altered in patients with PTSD include the hippocampus and amygdala as well as cortical regions including the anterior cingulate, insula, and orbitofrontal region. These areas interconnect to form a neural circuit that mediates, among other functions, adaptations to stress and fear conditioning. Changes in these circuits have been proposed to have a direct link to the development of PTSD.¹⁷

A hallmark feature of PTSD is reduced hippocampal volume. The hippocampus is implicated in the control of stress responses, declarative memory, and contextual aspects of fear conditioning. Prolonged exposure to stress and high levels of glucocorticoids in laboratory animals damages the hippocampus, leading to reduction in dendritic branching, loss of dendritic spines, and impairment of neurogenesis.¹⁸

The amygdala is a limbic structure involved in emotional processing and is critical for the acquisition of fear responses. The functional role of the amygdala in mediating both stress responses and emotional learning implicate its role in the pathophysiology of PTSD. Although there is no clear evidence for structural alterations of the amygdala in PTSD, functional imaging studies have revealed hyper-responsiveness of the amygdala in patients with PTSD during the presentation of stressful scripts, cues, and/or trauma reminders. PTSD patients further show increased amygdala responses to general emotional stimuli, such as emotional faces, that are not trauma-associated. The amygdala also seems to be

¹⁵ *Id.*

¹⁶ *Id.*

¹⁷ *Supra* note 6.

¹⁸ *Id.*

sensitized to the presentation of subliminally threatening cues in patients with PTSD, and increased activation of the amygdala has been reported in PTSD patients during fear acquisition in a fear conditioning experiment.¹⁹

The medial prefrontal cortex (PFC) comprises the anterior cingulate cortex (ACC), subcallosal cortex, and the medial frontal gyrus. The medial PFC exerts inhibitory control over stress responses and emotional reactivity in part by its connection with the amygdala. The medial PFC further mediates extinction of conditioned fear through active inhibition of acquired fear responses. Patients with PTSD exhibit decreased volumes of the frontal cortex, including reduced ACC volumes. This reduction in ACC volume has been correlated with PTSD symptom severity in some studies. Functional imaging studies have found decreased activation of the medial PFC in PTSD patients in response to stimuli, such as trauma scripts, combat pictures and sounds, trauma-unrelated negative narratives, fearful faces, emotional stroop, and others; although, there are also discordant findings. Reduced activation of the medial PFC was associated with PTSD symptom severity in several studies and successful SSRI treatment has been shown to restore medial prefrontal cortex activation patterns.²⁰

Core endocrine features of PTSD include abnormal regulation of cortisol and thyroid hormones, though there is some disagreement about these findings in the literature. Core neurochemical features of PTSD include abnormal regulation of catecholamine, serotonin, amino acid, peptide, and opioid neurotransmitters, each of which is found in brain circuits that regulate/integrate stress and fear responses. A cardinal feature of patients with PTSD is sustained hyperactivity of the autonomic sympathetic branch of the autonomic nervous system, as evidenced by elevations in heart rate, blood pressure, skin conductance, and other psychophysiological measures. Administration of the centrally acting β -adrenergic receptor antagonist propranolol shortly after exposure to psychological trauma has been reported to reduce PTSD symptom severity and reactivity to trauma cues.²¹

C. Effects on Health-Related Quality of Life.

Among patients, posttraumatic stress symptoms indicative of PTSD were associated with a considerable decrease in health-related quality of life. PTSD symptoms may, therefore, raise a major barrier for full recovery of even minor levels of severity in injury patients.²² Two years after injury, posttraumatic stress symptoms were associated with more problems on almost all domains of functional outcome and a considerable decrease in health-related quality of life in both non-hospitalized and hospitalized patients. We conclude that, among patients admitted to an emergency department due to injuries of all causes and severity levels, posttraumatic stress symptoms indicative of PTSD are associated with decreased health-related quality of life even after correction for possible confounders such as comorbidity. Hence, PTSD is a major barrier for full recovery of injury in patients with even minor levels of severity.

¹⁹ *Id.*

²⁰ *Id.*

²¹ *See Supra* note 6

²² Haagsma et al., *Post-traumatic Stress Symptoms and Health Related Quality of Life: a Two Year Follow-up Study of Injury Treated at the Emergency Department*

II. Conventional Treatments Prove Ineffective at Treating PTSD.

The standard treatment plan for treating PTSD is some combination—depending on the PTSD’s severity—of talk therapy (psychotherapy) and pharmacological therapy.²³ The most common talk therapy treatment used is cognitive-behavioral therapy (CBT).²⁴ CBT, of which there are different types, appears to be the most effective type of talk therapy for treating PTSD.²⁵ The purpose of CBT is to help the patients “understand and change how [they] think about [their] trauma and its aftermath” by confronting and discussing past traumatic events.²⁶ Other types of talk therapy exist; however, experts are uncertain about their efficacy.²⁷ Eye movement desensitization and reprocessing (EMDR) is one such treatment.²⁸ In EMDR, “while thinking of or talking about [their] memories, [the patients] focus on other stimuli like eye movements, hand taps, and sounds.”²⁹ Examples of other alternative therapies are group therapy and family therapy.³⁰

Antidepressant medication is the most common pharmacotherapy used to supplement talk therapy treatment.³¹ The most prevalent antidepressants are selective serotonin reuptake inhibitors (SSRI).³² SSRIs reduce PTSD symptoms by altering the chemical balance of patient’s brains through artificial increases in serotonin levels.³³ Examples of SSRIs are Prozac and Zoloft.³⁴ Serotonin-norepinephrine reuptake inhibitors (SNRIs) may be used as an alternative to SSRIs. If these antidepressants do not suppress the patient’s symptoms, then a doctor may prescribe tricyclic antidepressants.³⁵ If the patient does not respond to antidepressants in general, then a doctor may recommend Benzodiazepines.³⁶ Benzodiazepines are thought to be more useful for treating PTSD symptoms if immediate relief is needed because antidepressants take several weeks to take effect.³⁷ Other drugs may be prescribed for specific symptoms, such as antipsychotic and anti-anxiety medication.³⁸

Although there are a wide range of psychological and pharmacotherapies, half of PTSD sufferers go untreated and “[a]bout a third of PTSD victims never recover despite treatment.”³⁹ A 2010 Institute of Medicine (IOM) panel that examined this issue, “strongly

23 <http://health.nytimes.com/health/guides/disease/post-traumatic-stress-disorder/print.html>.

24 *Id.*

25 <http://www.ptsd.va.gov/public/pages/treatment-ptsd.asp>

26 *Id.*

27 *Id.*

28 *Id.*

29 *Id.*

30 *Id.*

31 <http://health.nytimes.com/health/guides/disease/post-traumatic-stress-disorder/print.html>

32 *Id.*

33 <http://www.ptsd.va.gov/public/pages/treatment-ptsd.asp>

34 *Id.*

35 <http://health.nytimes.com/health/guides/disease/post-traumatic-stress-disorder/print.html>

36 *Id.*

37 *Id.*

38 *Id.*

39 <http://usatoday30.usatoday.com/news/military/story/2012-07-13/post-traumatic-stress-disorder-programs/56207754/1>

avored psychotherapy intervention, which has been proven by research and clinical use, and was less positive about drug or alternative therapies, which lack scientific studies to support their effectiveness."⁴⁰ Furthermore, a 2008 IOM panel "concluded that neither (selective serotonin inhibitors) nor any other drugs could be considered effective for the treatment of PTSD. The evidence base . . . [for] pharmacotherapy for PTSD is at best mixed and inconclusive."⁴¹ The 2010 panel affirmed that "more research is necessary."⁴² In addition, Professor Sue Sisley at the University of Arizona has said with respect to PTSD that drugs "like Zoloft and Paxil have proven entirely inadequate."⁴³ *A double standard exists when the threshold to prove marijuana's efficacy in treating PTSD is so high while ineffective, pharmaceutical drugs are rampantly accessible and freely prescribed.*

While the efficacy of pharmacotherapy is uncertain, its side effects are painfully clear. SSRIs can "cause agitation, nausea, and diarrhea. Sexual function side effects include low sex drive, inability to have an orgasm, and impotence."⁴⁴ Furthermore, there "have been many concerns about SSRIs and increased risk of suicidal behavior."⁴⁵ SNRIs also may impair sexual function and can increase blood pressure and heart rate.⁴⁶ Side effects of tricyclic antidepressants "include sleep disturbance, abrupt reduction in blood pressure upon standing, weight gain, sexual dysfunction, and mental disturbance."⁴⁷ Benzodiazepines have a risk of dependency and abuse and can cause daytime drowsiness, a hung-over feeling, and agitation.⁴⁸ Generally speaking all of these drugs should be closely monitored if used by the elderly, heavy drinkers, those with heart conditions, glaucoma, liver and kidney problems, or other health issues as death may result.⁴⁹ Pregnant woman should avoid using these drugs as birth defects may result.⁵⁰ Many patients have also reported severe withdrawal symptoms for many of these drugs, including dizziness, nausea, anxiety, and insomnia sometimes lasting one to three weeks.⁵¹

As a result of the uncertainty surrounding pharmacotherapy's efficacy and the presence of numerous side effects, many PTSD victims have turned to medical marijuana for relief. Dr. Phil Leveque, a World War II veteran, is one such example of the shift toward medical marijuana.⁵² Dr. Leveque had his medical license revoked for issuing an estimated 1,000 medical marijuana permits to veterans suffering from PTSD.⁵³ According to Dr. Leveque, "Whether they were World War II, Korea, Vietnam or vets from the current

⁴⁰ *Id.*

⁴¹ *Id.*

⁴² *Id.*

⁴³ <http://www.theatlantic.com/health/archive/2012/01/the-case-for-treating-ptsd-in-veterans-with-medical-marijuana/251466/>

⁴⁴ <http://health.nytimes.com/health/guides/disease/post-traumatic-stress-disorder/print.html>

⁴⁵ *Id.*

⁴⁶ *Id.*

⁴⁷ *Id.*

⁴⁸ *Id.*

⁴⁹ *Id.*

⁵⁰ *Id.*

⁵¹ *Id.*

⁵² <http://www.theatlantic.com/health/archive/2012/01/the-case-for-treating-ptsd-in-veterans-with-medical-marijuana/251466/>

⁵³ *Id.*

conflicts, 100 percent of my patients said it was better than any drug they were prescribed for PTSD.”⁵⁴

III. Studies Demonstrate Marijuana’s Efficacy in Treating PTSD Symptoms.

Again, the Colleges’ Evidence Review of scientific studies addressing the benefits and harms of cannabis therapy for PTSD excluded articles that contained: “animal studies, or experiments on biochemical or pathophysiological pathways; case reports or case series; editorials or opinions; [anything] not addressing a key question.” This approach, coupled with NIDA’s research-prohibitive agenda, effectively eliminates any and all scientifically-controlled studies that directly test the efficacy of marijuana in treating PTSD. As a result of the exclusions, the Colleges were unable to find a single study that was on point, including the research presented in this petition. Hence, the recommendation of the Medical Advisory Committee to the director of ADHS in 2012 was to not include PTSD because “at this time, there is insufficient valid, scientific evidence.” We believe this recommendation was based on incomplete information.

The following studies directly address the efficacy of marijuana in treating PTSD. These studies represent reasonable, objective scientific data that should lead to reasonable conclusions. We hope that ADHS will consider this research when making its determination of whether to list PTSD as a qualifying condition under the AMMA.

A. The University of Haifa Studies Provide Direct Scientific Evidence of Marijuana’s Potential Efficacy in Treating PTSD.⁵⁵

The University of Haifa’s 2011 Study—*The Role of Cannabinoids in Modulating Emotional and Non-Emotional Memory Processes in the Hippocampus*—found that the administration of cannabinoids (the active compounds found in medicinal marijuana) after experiencing a traumatic event blocks the development of post-traumatic stress (PTSD)-like symptoms in rats.⁵⁶ The rats were divided into four groups. One group was given no marijuana; another was given a marijuana injection two hours after being exposed to a traumatic event; the third group after 24 hours and the fourth after 48. The rats were examined a week later and—while the group that was injected with marijuana 48 hours or more after trauma continued to display PTSD symptoms as well as a high level of anxiety—the PTSD symptoms disappeared in rates that were given marijuana two or 24 hours after experiencing trauma. The Study’s author, Dr. Akirav, concluded that *the results suggest that, while cannabis does not erase the experience of trauma, marijuana specifically prevented the development of post-traumatic symptoms.*⁵⁷

⁵⁴ *Id.*

⁵⁵ For full study refer to Exhibit A.

⁵⁶ Akirav, Irit, THE ROLE OF CANNABINOIDS IN MODULATING EMOTIONAL AND NON-EMOTIONAL MEMORY PROCESSES IN THE HIPPOCAMPUS (2011), available at, http://www.frontiersin.org/Behavioral_Neuroscience/10.3389/fnbeh.2011.00034/full#B155 (hereinafter, 2011 Haifa Study).

⁵⁷ The results also suggest a required window within which the treatment must be administered.

i. Fear Retrieval.

More specifically, the Haifa Study states that “WIN 55,212-2 (5 µg) (a cannabinoid) injected into the dorsal hippocampus increases the number of reference memory errors in the eight-arm radial-maze task, suggesting impairment of memory retrieval.⁵⁸ To give further background, the article provides that “post-training intrahippocampal administration of WIN 55,212-2 (2.5 and 5 µg) disrupts long-term spatial memory, but not acquisition or short-term memory, in a rat reference memory task in the water maze.”⁵⁹ Additionally,

[i]n neuronal circuits, memory storage depends on activity-dependent modifications in synaptic efficacy, such as long-term potentiation (LTP) and long-term depression (LTD), which are the two main forms of synaptic plasticity in the brain. *Cannabinoid receptor activation inhibits both LTP and LTD induction in the hippocampal slice.* The inhibition of LTP in field potentials in the CA1 region has been demonstrated using THC, HU-210, WIN 55,212-2, 2-AG, and anandamide⁶⁰ and has been found recently to inhibit hippocampal LTD of CA1 field potentials as well.⁶¹

*Simply put, the research cited suggests that cannabis can prevent the hippocampus from retrieving traumatic memories.*⁶²

ii. Fear Extinction.

The Haifa Study further states, “Although considerable evidence suggests that activation of CB₁ (cannabinoid) receptors can induce learning and memory impairments,⁶³ CB₁ receptors are essential for the extinction of conditioned fear associations,⁶⁴ indicating an important role for this receptor in neuronal emotional learning and memory.”

Extinction was established as a tool to treat conditioned fear by Freud in the 1920s. It has become widely accepted that a deficit in the capacity to extinguish memories of fear is at the root of fear disorders as a result of the distinction between those who do and do not develop serious symptoms after fearsome experiences, and the fact that fear disorders are treated with therapy based on extinction procedures. Moreover, panic attacks, phobias, and

⁵⁸ *Id.* (citing Wegener et al., 2008).

⁵⁹ *Id.* (citing Yim et al., 2008).

⁶⁰ *Id.* (citing Nowicky et al., 1987; Collins et al., 1994, 1995; Terranova et al., 1995; Misner and Sullivan, 1999).

⁶¹ *Id.* (citing Misner and Sullivan, 1999) (emphasis added).

⁶² A team at National Cheng-Kung University’s Institute of Basic Medical Sciences and Department of Pharmacology in Taiwan demonstrated “that bilateral infusion of CB₁ receptor agonists into the amygdala after memory reactivation blocked reconsolidation of fear memory measured with fear-potentiated startle.” Lin, Hui-Ching, Mao Sheng-Chun, and Gean, Po-Wu, EFFECTS OF INTRA-AMYGDALA INFUSION OF CB₁ RECEPTOR AGONISTS ON THE RECONSOLIDATION OF FEAR-POTENTIATED STARTLE (January 11, 2013), available at <http://www.learnmem.org/cgi/doi/10.1101/lm.217006>. For full study refer to Exhibit F.

Additionally, a Brazilian team concluded that “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. Pamplona, Fabrício et al., THE CANNABINOID RECEPTOR AGONIST WIN 55,212-2 FACILITATES THE EXTINCTION OF CONTEXTUAL FEAR MEMORY AND SPATIAL MEMORY IN RATS, Springer-Verlag (2006). For full study refer to Exhibit G.

⁶³ *Supra* note 56 (citing Sullivan, 2000; Robinson et al., 2003; O’Shea et al., 2004; Varvel et al., 2005).

⁶⁴ *Id.* (citing Marsicano et al., 2002).

particularly post-traumatic stress disorder (PTSD) are viewed by many as a deficit of extinction that should therefore be treated by an intensification of extinction.⁶⁵

Dr. Akirav cites other studies demonstrating “that pharmacological activation of eCB (endocannabinoid system) signaling promotes extinction of fear memories.”⁶⁶ The procedure used to demonstrate the effects of the WIN 55,212-2 cannabinoid on extinction “is dependent on both the amygdala and hippocampus as a single CS–US (context–footshock) pairing establishes a robust long-term memory, expressed as an increase in latency to enter the dark chamber at testing.” Here, “the results of Marsicano et al. (2002)⁶⁷ and subsequent investigations demonstrate that inhibition of eCB transmission robustly inhibits (or prolongs) fear extinction.⁶⁸ *Conversely, stimulation of eCB transmission accelerates fear extinction.*”⁶⁹

In a separate study at the University of Haifa, Drs. Akirav and Ganon-Elazar found “preclinical support to the suggestion that cannabinoids could represent a target for the treatment of diseases associated with the inappropriate retention of aversive memories such as posttraumatic stress disorder.”⁷⁰ In the 2009 Haifa Study, Ganon-Elazar and Akirav demonstrated the following: (1) the effects of WIN55,212-2 could not be attributed to sensorimotor deficits, because these parameters seemed unchanged by WINN 55,212-2 microinjected into the BLA; and (2) the CB₁ receptor in the BLA is crucially involved in the extinction of IA, because the CB₁ receptor antagonist AM251 microinjected into the BLA significantly blocked extinction.⁷¹ Together, these studies support a therapeutic application for cannabinoids in extinguishing fears for conditions associated with aversive memory retention.

iii. Stress Mediation.

Furthermore, “considerable evidence suggests that cannabinoids are anxiolytics which modulate the behavioral and physiological response to stressful events.”⁷² Consequently, the effects of CB₁ agonists on learning and memory may be attributable to a general modulation of anxiety or stress levels and not to memory *per se*. The hippocampus is often implicated in the neurobiology of stress.⁷³ Thus, in PTSD and major depression

⁶⁵ *Id.* (citing Charney et al., 1993; Wessa and Flor, 2007; Milad et al., 2008).

⁶⁶ *Id.* “For example, Chhatwal et al. (2005) found that systemic administration of the eCB transporter AM404 (10 mg/kg) promotes extinction of fear that was conditioned using fear-potentiated startle.” *Id.*

⁶⁷ *See infra* Part III.C.

⁶⁸ *Id.* (citing Suzuki et al., 2004; Pamplona et al., 2006; Ganon-Elazar and Akirav, 2009; Abush and Akirav, 2010).

⁶⁹ *Id.* (citing Suzuki et al., 2004; Chhatwal et al., 2005; Barad et al., 2006; Abush and Akirav, 2010) (emphasis added).

⁷⁰ Ganon-Elazar, Eti and Akirav, Irit, CANNABINOID RECEPTOR ACTIVATION IN THE BASOLATERAL AMYGDALA BLOCKS THE EFFECTS OF STRESS ON THE CONDITIONING AND EXTINCTION OF INHIBITORY AVOIDANCE. 29(36) J. NEUROSCI at 11078-11088 (September 9, 2009) (hereinafter, 2009 Haifa Study). For full study refer to Exhibit E.

⁷¹ *Id.*

⁷² *Supra* note 56 (citing Viveros et al., 2007; Hill et al., 2010).

⁷³ *Id.*

patients, hippocampus volumes are reduced,⁷⁴ and smaller hippocampal volumes are predictive of vulnerability to developing stress-related disorders.⁷⁵

Finally, [r]esults from many studies indicate that the eCB (endocannabinoid) system modulates unconditioned stress- and anxiety-like responses.⁷⁶ A general conclusion that can be tentatively derived from the complicated and often contradictory literature is that inhibition of eCB signaling increases stress and anxiety, while moderate increases in eCB signaling decrease stress and anxiety.⁷⁷ For anxiety, “these studies suggest that eCBs act at CB₁ receptors to reduce anxiety” when taken together.⁷⁸ Regarding fear generally, the Study concludes, “Overall it appears that, as in the case of unconditioned fear, inhibition of eCB transmission increases fear while moderate stimulation of eCB transmission decreases fear.”⁷⁹

In the 2011 Haifa Study, “[t]echniques based on intracranial injections of cannabinoids in rats revealed that activation of CB₁ receptors is involved in inducing anxiolytic- or antidepressant-like effects.⁸⁰ For example, Rubino et al. (2008a) found that low doses of THC microinjected into the PFC (10 µg) or ventral hippocampus (5 µg) in rats induces an anxiolytic-like response during tests in the EPM, while higher doses do not show an anxiolytic effect and even seem to switch into an anxiogenic profile. To summarize the Haifa Study’s results regarding “the role of the eCB system in stress, anxiety, and conditioned fear, there is a general consensus that the effects of cannabinoid agonists on anxiety seem to be biphasic, with low doses being anxiolytic.”⁸¹ The 2009 Haifa Study also stated, “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.” In conclusion, the Haifa Studies demonstrate that marijuana can help treat the underlying causes of PTSD symptoms in patients.

B. Fraser Found that Naboline, a Cannabinoid, Greatly Reduced or Abolished Nightmares in PTSD Sufferers.⁸²

Fraser’s article—*The Use of a Synthetic Cannabinoid in the Management of Treatment-Resistant Nightmares in Posttraumatic Stress Disorder (PTSD)*—states:

⁷⁴ *Id.* (citing Bremner et al., 1995; Sheline et al., 1999; Woon and Hedges, 2008).

⁷⁵ *Id.* (citing Pitman et al., 2006).

⁷⁶ *Id.* (citing Viveros et al., 2005; Gorzalka et al., 2008; Lutz, 2009); Resstel, Leonardo, *5-HT_{1A} Receptors are Involved in the Cannabidiol-Induced Attenuation of Behavioral and Cardiovascular Responses to Acute Restraint Stress in Rats*, *BR. J. CLIN. PHARMACOL* 156, 181–188 (2009) (concluding that “present findings indicate that CBD, by activating 5-HT_{1A} receptors, can attenuate physiological and behavioral responses to restraint stress” and stating, “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.”).

⁷⁷ *Id.* (citing Lutz, 2009 at Table 2).

⁷⁸ *Supra* note 56.

⁷⁹ *Id.* (emphasis added).

⁸⁰ *Id.* (citing Bambico et al. 2007, Moreira et al., 2007; Rubino et al., 2008a,b)

⁸¹ *Supra* note 56. To be fair, the Study also found that high doses could be axiogenic. *Id.*

⁸² For full study refer to Exhibit B.

"Endocannabinoids are thought to exert an effect through a variety of interactions with the CNS (central nervous system) related to PTSD . . . including "the function of the hippocampus and amygdala, and control of cortical regulation of memory processes.⁸³ Fraser prepared this open-label clinical trial "to evaluate the effects of nabilone, an endocannabinoid receptor agonist, on treatment-resistant nightmares in patients diagnosed with post-traumatic stress disorder (PTSD)." Fraser's study directly addresses marijuana's potential to treat symptoms of PTSD.

The following quotes Fraser's summary of his methods results:

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 night sweats 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.⁸⁴

Fraser found that 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."⁸⁵

C. Marsicano Found that the Endogenous Cannabinoid System Could Therapeutically Treat Diseases such as Post-traumatic Stress Disorder.⁸⁶

In *The Endogenous Cannabinoid System Controls Extinction of Aversive Memories*, Marsicano, et al. begins, "Acquisition and storage of aversive memories is one of the basic principles of central nervous systems throughout the animal kingdom."⁸⁷ This team of researchers "show[ed] that the endogenous cannabinoid system has a central function in extinction of aversive memories."⁸⁸ Methods:

To study the involvement of the endogenous cannabinoid system in memory processing, we (the team) generated CB1-deficient mice. CB1^{2/2} mice and CB1^{Δ/Δ} littermates were tested in auditory fear conditioning, which is highly dependent on the amygdala and enables the dissection of different phases of memory formation, including acquisition, consolidation and

⁸³ George A. Fraser, THE USE OF A SYNTHETIC CANNABINOID IN THE MANAGEMENT OF TREATMENT-RESISTANT NIGHTMARES IN POSTTRAUMATIC STRESS DISORDER (PTSD) (2009).

⁸⁴ *Id.* (emphases added).

⁸⁵ *Id.* (emphases added).

⁸⁶ For full study refer to Exhibit C.

⁸⁷ Giovanni Marsicano et al., THE ENDOGENOUS CANNABINOID SYSTEM CONTROLS EXTINCTION OF AVERSIVE MEMORIES (2002) (citing LeDoux, J. E., *Emotion Circuits in the Brain*, 23 ANNU. REV. NEUROSCI., 155-184 (2000)).

⁸⁸ Giovanni Marsicano et al., THE ENDOGENOUS CANNABINOID SYSTEM CONTROLS EXTINCTION OF AVERSIVE MEMORIES (2002) (emphases added).

extinction . . . As the amygdala has a crucial role for extinction of aversive memories,⁸⁹ [the team] studied amygdala-dependent memory performance in the absence of possible confounding influences of the hippocampus by re-exposing the mice to the tone in an environment different from the conditioning context.⁹⁰

The Marsicano team “demonstrated a specific involvement of CB1-mediated neurotransmission in extinction of aversive memories.”⁹¹ The Study stated that “[i]t remains to be shown whether CB₁ is not only involved in extinction of aversive memories but also in adaptation to aversive situations in general and/or in extinction of memories, independently from their emotional value.” Despite this, the team concluded that its “findings suggest that *the endogenous cannabinoid system could represent a therapeutic target for the treatment of diseases associated with inappropriate retention of aversive memories or inadequate responses to aversive situations, such as posttraumatic stress disorders*⁹², *phobias, and certain forms of chronic pain.*”⁹³

The above studies should provide sufficient support for marijuana’s efficacy in treating PTSD. Furthermore, Dr. Sue Sisley’s research⁹⁴ found numerous anecdotal reports from combat veterans and from other first responders, like policemen and firemen, discussing the value of cannabis in managing their PTSD symptoms.⁹⁵ Dr. Sisley also points out that marijuana can treat PTSD’s host of symptoms by itself instead of having to prescribe five or six synthetic drugs that each have different side effects.

Because of NIDA’s obstruction to approved marijuana-related research, Dr. Sisley’s anecdotal evidence and the studies summarized in this Part III provide the best direct evidence addressing the issue at hand. Despite the legislature’s attempts to open the doors to increased marijuana-related research at universities through SB 1443, NIDA’s research monopoly will continue to reign. Our research finds that marijuana can potentially treat the symptoms and underlying causes of PTSD effectively; thus, ADHS should amend the AMMA and add PTSD as a qualifying condition.

IV. Listing PTSD as a Qualifying Condition under the AMMA Allows Sufferers the Proper Freedom to Treat their Debilitating Conditions.

Ultimately, the word freedom properly captures the issue of whether the AMMA should list PTSD as a debilitating condition. The many sufferers of PTSD that are veterans spend swaths of their days needlessly reliving traumatic combat experiences endured

⁸⁹ *Id.* (citing Falls, W. A., Miserendino, M. J. & Davis, M., *Extinction of Fear-Potentiated Startle: Blockade by Infusion of an NMDA Antagonist into the Amygdala*, 12 J. NEUROSCI. at 854–863 (1992) and Lu, K. T., Walker, D. L. & Davis, M., *Mitogen-Activated Protein Kinase Cascade in the Basolateral Nucleus of Amygdala is Involved in Extinction of Fear-Potentiated Startle*, J. NEUROSCI. 21 RC162 (2001)).

⁹⁰ *Id.* (citing LeDoux, J. E., *Emotion Circuits in the Brain*, ANNU. REV. NEUROGL., 23, 155–184 (2000)).
⁹¹ *Supra* note 73.

⁹² *Id.* (citing Davis, M., Falls, W. A. & Gerwitz, J. in *Contemporary Issues in Modeling Psychopathology* (eds. Myslobodsky, M. S. & Weiner, I.) 113–141 (Kluwer Academic, Norwell, 2000).

⁹³ *Id.* (citing Pertwee, R. G., *Cannabinoid Receptors and Pain*, PROG. NEUROBIOL. 63, 569–611 (2001).

⁹⁴ For study refer to Exhibit D.

⁹⁵ http://www.maps.org/media/view/dr_sue_sisley_talks_about_medical_marijuana_ptsd_and_scientific_freedom.

while fighting for our freedoms. These returning veterans do not, however, have the full freedom to choose natural, effective remedies like marijuana instead of side-effect ridden pharmaceutical drugs to treat their PTSD. Patient autonomy demands that our veterans receive the freedom to choose how they will treat the wounds, both mental and physical, of their fight abroad to preserve our freedom. Given the recent end to American involvement in the War in Iraq and the growing number of troops returning home from Afghanistan each day, there is no better time than now to add PTSD as a qualifying condition to the AMMA.⁹⁶ The more ADHS delays, the more Arizonans with PTSD will endure unconscionable, chronic pain and suffering. ADHS should make the right decision for Arizonan PTSD sufferers by recommending to add PTSD as a qualifying condition to the AMMA.

⁹⁶ A recent VA study revealed that approximately 22 veterans are committing suicide per day as a result of their inability to deal with the after-effects of a brutal war. Greg Jaffe, *VA Study Finds More Veterans Committing Suicide* (January 31, 2013), available at http://www.washingtonpost.com/national/va-study-finds-more-veterans-committing-suicide/2013/01/31/1092b330-5a68-11e2-9fa9-5fbd9530eb9_story.html.

Exhibit G

STUDY

*The Cannabinoid Receptor Agonist WIN
55,212-2 Facilitates the Extinction of
Contextual Fear Memory and Spatial Memory
in Rats*

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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 ($23\pm 2^\circ\text{C}$) 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]pyrrol[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 stop-watches 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

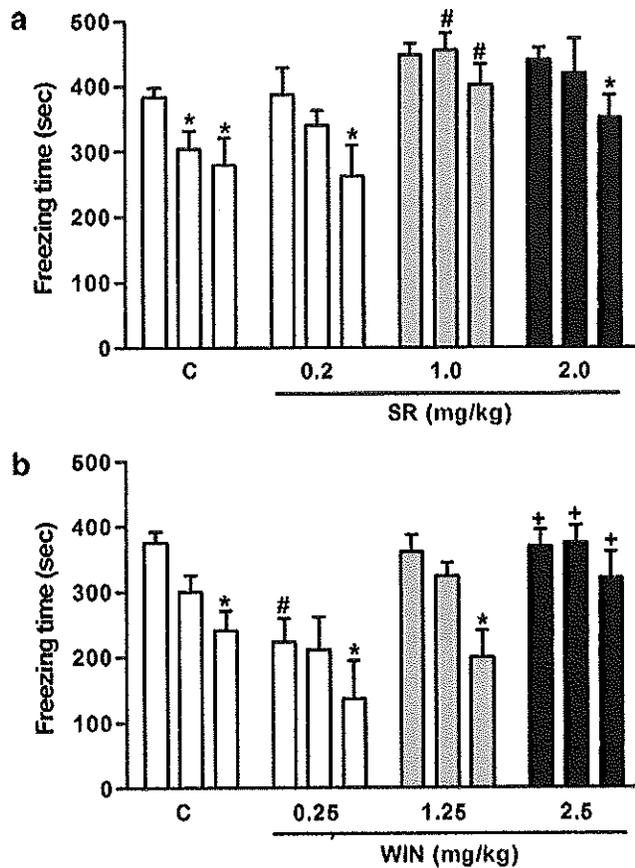


Fig. 1 Effects of the selective CB1 cannabinoid receptor antagonist SR (0.2, 1.0 or 2.0 mg/kg, i.p.) and cannabinoid agonist WIN (0.25, 1.25, or 2.50 mg/kg, i.p.) on the extinction of recent contextual fear memory in rats. Data are expressed as mean±SEM of the time spent freezing expressed by SR-treated 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 [$F(4,29)=13.62, p<0.0001$] and trials [$F(4,116)=18.02, p<0.00001$], but no treatment \times 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

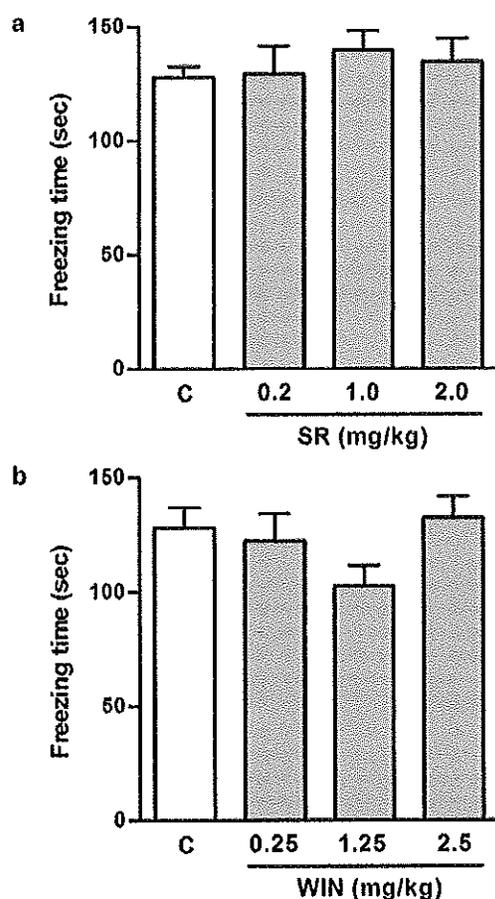


Fig. 2 Effects of the selective CB1 cannabinoid receptor antagonist SR (0.2, 1.0, or 2.0 mg/kg, i.p.) and cannabinoid agonist WIN (0.25, 1.25, or 2.50 mg/kg, i.p.) on the retrieval of recent contextual fear memory in rats. Data are expressed as mean±SEM of the time spent freezing expressed by SR-treated 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 $n=8$)

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

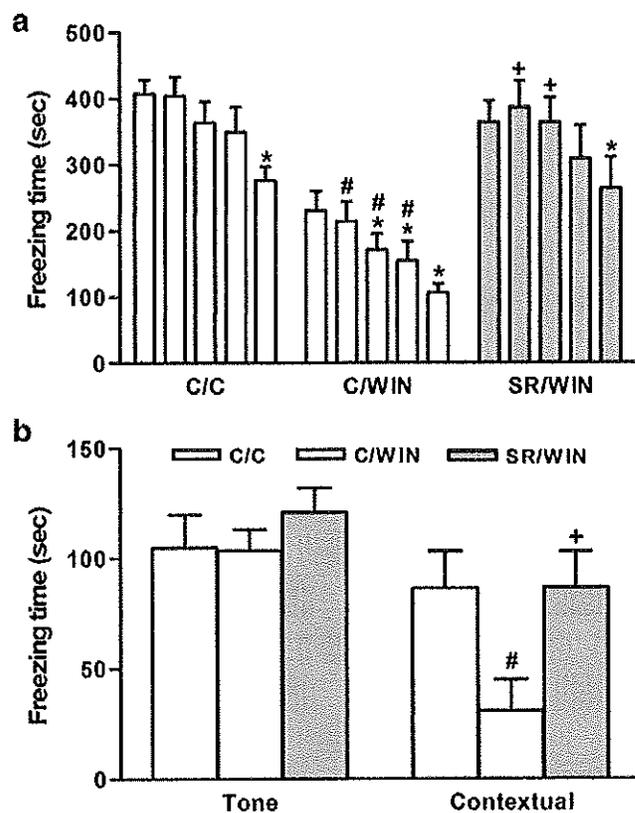


Fig. 3 Effects of the cannabinoid agonist WIN (0.25 mg/kg, i.p.) and pretreatment with the selective CB1 cannabinoid receptor antagonist SR (0.2 mg/kg, i.p.) on extinction of remote contextual fear memory in rats. The animals received one injection of SR or control solution (c) followed by one injection 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 $n=11$)

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 \times 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

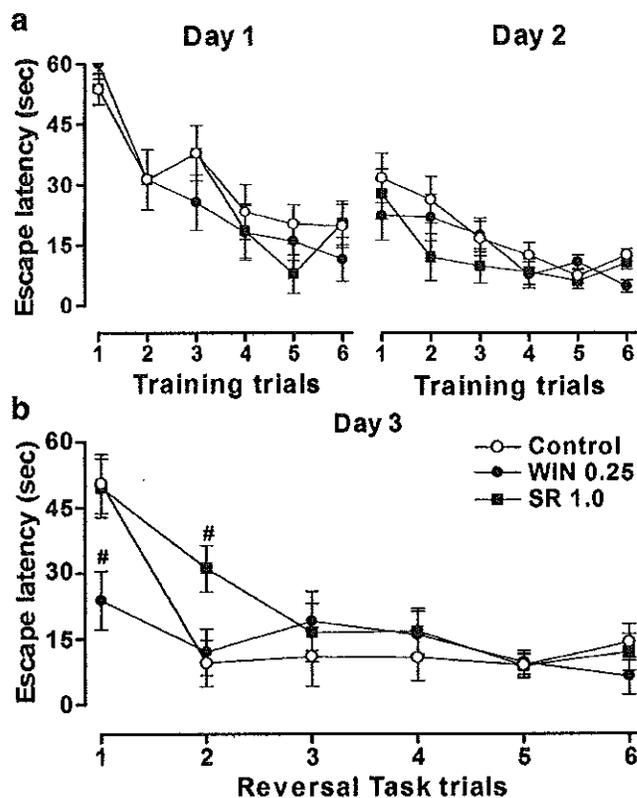


Fig. 4 Effects of the selective CB1 cannabinoid receptor antagonist SR (1.0 mg/kg, i.p.) and cannabinoid agonist WIN (0.25 mg/kg, i.p.) on the performance of rats in the water maze reversal task. **a** The animals were trained to find a submerged platform in a fixed position during six trials on two 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 $n=8$)

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.

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).

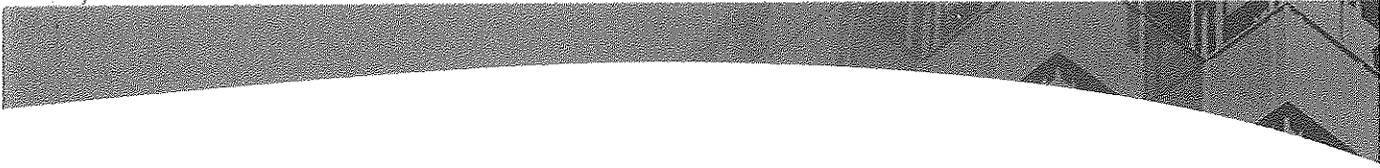
In conclusion, the present results reinforce those of previous studies demonstrating that the disruption of CB1 cannabinoid receptor signaling impairs the extinction of both conditioned fear and spatial memory in rodents. More importantly, our results suggest that the extinction of contextual fear memory 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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**ARIZONA CANNABIS
NURSES ASSOCIATION**

JUL 25 2013

**Heather Manus, RN,
President**

July 24, 2013

Hon. Will Humble,
Director
Arizona Department of Health Services
State of Arizona
PO Box 19000
Phoenix, AZ 85005

Re: Request to Add a Debilitating Medical Condition or Treatment – Post Traumatic Stress Disorder (“PTSD”) – Submitted by the Arizona Cannabis Nurses Association (“AZ CNA”)

Dear Director Humble:

On behalf of the Arizona Cannabis Nurses Association, we respectfully request that the Arizona Department Health Services consider and approve the enclosed Application and specified supporting materials submitted herewith in order to add a debilitating medical condition – PTSD – to the list of debilitating medical conditions for the Arizona Medical Marijuana Program.

Please find enclosed the following:

- A Request to List Post-Traumatic Stress Disorder (PTSD) as a Qualifying Condition under the Arizona Medical Marijuana Act (AMMA)
- A description of the symptoms and other physiological effects experienced by an individual suffering from PTSD or a treatment of PTSD that may impair the ability of the individual to accomplish activities of daily living;
- The availability of conventional medical treatments to provide therapeutic or palliative benefits for PTSD or a treatment of the PTSD;
- A summary of the evidence that the use of marijuana will provide therapeutic or palliative benefits for PTSD or a treatment of PTSD; and
- Exhibits A-G, including articles, published in peer-reviewed, scientific journals reporting the results of research concerning marijuana’s effects on or treatment of PTSD, and supporting why PTSD should be added to the list of medical conditions approved for treatment through marijuana use.

A favorable ruling on the issue of PTSD is of particular concern to our returning war heroes – veterans of the Iraq and Afghanistan wars. While they courageously fought for the freedom of others, these returning veterans do not, however, have the full freedom to choose natural, effective remedies like medical cannabis instead of side-effect ridden pharmaceutical drugs to treat their PTSD.

The VA has reported that suicides among the active and retired military are at an all-time high. Nearly, one (1) suicide per day for active members, and twenty-two (22) per day for other veterans. (Greg Jaffe, VA Study Finds More Veterans Committing Suicide (January 31, 2013).

One study recently estimated that nearly a third of those returning from combat in Iraq and Afghanistan have symptoms of PTSD.

Three states have recently ratified or approved medical marijuana as a PTSD related debilitating condition. New Mexico recently decided that PTSD would remain a qualifying condition under its medical marijuana program. Oregon and Maine, even more recently approved PTSD as a qualifying condition under their medical marijuana programs.

In my career as a registered psychiatric nurse from New Mexico, I have cared for many veterans who successfully use medical cannabis to assist with the array of symptoms associated with PTSD and other combat related injuries. Time and time again, PTSD patients who use cannabis prove to have an increased quality of life and reduced need for pharmaceutical medications. Most importantly, by including PTSD to the list of qualifying conditions, Veterans have the freedom to choose the medication that works best for them. As an RN, I have seen repeated cases and clinical evidence proving cannabis to be a gentle plant medicine that provides great benefit with minimal adverse effects. Our Veterans fought for the freedoms we enjoy. Let's show our support by allowing them the right to choose effective medicine that increases their personal health and well-being.

ADHS should follow the example of the other states who recognize PTSD as a legitimate debilitating condition which is treated effectively and compassionately with medical cannabis. We should allow nothing less for the returning war heroes and veterans who reside in Arizona.

Thank you in advance for your careful consideration. Should you have any questions, comments or concerns, please do not hesitate to contact me.

Sincerely,

Heather Manus, R.N.
President, Arizona Cannabis Nurses Association

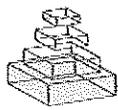
Exhibit A

STUDY

*The Role of Cannabinoids in Modulating
Emotional and Non-emotional Memory
Processes in the Hippocampus*

By: Dr. Irit Akirav
Department of Psychology
University of Haifa, Haifa, Israel

June 23, 2011



REVIEW ARTICLE

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Front. Behav. Neurosci., 2011 | Volume 5 | Article ID 10034 | doi:10.3389/fnbeh.2011.00034

The role of cannabinoids in modulating emotional and non-emotional memory processes in the hippocampus

Irit Akirav¹¹Department of Psychology, University of Haifa, Haifa, Israel

Cannabinoid agonists generally have a disruptive effect on memory, learning, and operant behavior that is considered to be hippocampus-dependent. Nevertheless, under certain conditions, cannabinoid receptor activation may facilitate neuronal learning processes. For example, CB₁ receptors are essential for the extinction of conditioned fear associations, indicating an important role for this receptor in neuronal emotional learning and memory. This review examines the diverse effects of cannabinoids on hippocampal memory and plasticity. It shows how the effects of cannabinoid receptor activation may vary depending on the route of administration, the nature of the task (aversive or not), and whether it involves emotional memory formation (e.g., conditioned fear and extinction learning) or non-emotional memory formation (e.g., spatial learning). It also examines the memory stage under investigation (acquisition, consolidation, retrieval, extinction), and the brain areas involved. Differences between the effects of exogenous and endogenous agonists are also discussed. The apparently biphasic effects of cannabinoids on anxiety is noted as this implies that the effects of cannabinoid receptor agonists on hippocampal learning and memory may be attributable to a general modulation of anxiety or stress levels and not to memory *per se*. The review concludes that cannabinoids have diverse effects on hippocampal memory and plasticity that cannot be categorized simply into an impairing or an enhancing effect. A better understanding of the involvement of cannabinoids in memory processes will help determine whether the benefits of the clinical use of cannabinoids outweigh the risks of possible memory impairments.

Introduction

Considerable evidence suggests that cannabinoids impair hippocampal-dependent learning and memory processes, such as spatial learning and context-related memory tasks (Sullivan, 2000; Riedel and Davies, 2001). In this review, I will provide evidence that suggests that the effects of cannabinoids on memory and plasticity are complex and depend on several factors, such as the nature of the task (emotional or non-emotional), the memory stage investigated (acquisition, retrieval, and extinction), and the experimental model used. Naturally, the behavioral effects of cannabinoids on memory may vary as a function of dose, route of administration, and the specific drug used.

Cannabinoid Receptors in the Hippocampus

Cannabis has a long history of consumption both for recreational and medicinal uses. The main psychoactive constituent of marijuana, delta-9-tetrahydrocannabinol (THC), was identified in 1964 (Gaoni and Mechoulam, 1964) and this discovery led to the identification of the endogenous endocannabinoid (eCB) system. This system includes cannabinoid receptors (CB₁ and CB₂), eCBs [anandamide and 2-arachidonoyl-glycerol (2-AG)], enzymes involved in their synthesis and metabolism [fatty acid amide hydrolase (FAAH) for anandamide and the monoacylglycerol lipase (MAGL) for 2-AG], and an eCB transporter (Deyme et al., 1997; Freund et al., 2003; Kogan and Mechoulam, 2006). Recent cDNA cloning of the key enzymes such as *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL) accelerated molecular biological studies on the eCB biosyntheses (Bisogno et al., 2003; Okamoto et al., 2003). eCBs are synthesized “on demand” at the post-synaptic sites of neurons after an increase in neural activity and calcium ion influx, and are then released into the synaptic cleft. Their main function appears to be the suppression of neurotransmitter release from the presynapse. Thus, eCBs act as retrograde neurotransmitters, modulating other neurotransmitter systems.

CB₁ and CB₂ are metabotropic receptors coupled to G-proteins of the Gi/o type. CB₁ receptors are localized mainly in the central nervous system, but are also present in a variety of peripheral tissues; they are among the most abundant and widely distributed G-protein coupled receptors in the brain. CB₁ receptors are expressed in multiple brain areas, including the olfactory bulb, neocortex, pyriform cortex, hippocampus, amygdala, basal ganglia, thalamic and hypothalamic nuclei, cerebellar cortex, and brainstem nuclei (Harkoulomb et al., 1999, 2001; Katona et al., 2001). CB₂ receptors are mostly peripherally located on immunological tissues, but they have also been found within the central nervous system on neurons and glial cells with their expression mainly related to conditions of inflammation (Calleague et al., 2005; Nehat et al., 2005; Begg et al., 2006). More recent immunohistochemical analyses have revealed the presence of CB₂ receptors in apparently neuronal and glial processes in diverse rat brain areas, including the cerebellum and hippocampus (Van Nieuwenhuijzen et al., 2007; Orzioli et al., 2008).

In the hippocampus, CB₁ receptors are expressed at an especially high density in the dentate gyrus, CA1, and CA3 regions (Harkoulomb et al., 1999, 2001; Matsuda et al., 1999; Zhou et al., 1999). CB₁ receptors are predominantly localized on the axon terminals and preterminal segments of cholecystikinin (CCK)-expressing GABAergic interneurons (Nayiri et al., 2003); however, they have also been demonstrated to inhibit glutamatergic transmission in cultured hippocampal cells (Shen et al., 1996). CB₁ receptors located on GABAergic axon terminals are activated by lower concentrations of cannabinoid receptor agonists than CB₁ receptors located on glutamatergic terminals (Ono Shosaku et al., 2001; Hoffman et al., 2007) and CB₁ receptor expression is significantly lower on glutamatergic terminals than on GABA axon terminals in the hippocampus (Katona et al., 2006; Kawamura et al., 2006). Specifically, activation of hippocampal CB₁ receptors decreases GABA release (Katona et al., 1999; Hajos et al., 2000; Hoffman and Lupica, 2000; Hoffman et al., 2007). The CB₁-containing GABAergic interneurons are thought to control oscillatory electrical activity in the hippocampus in the theta and gamma frequencies, which plays a role in synchronizing pyramidal cell activity (Hoffman and Lupica, 2000).

Overall, the evidence favors a predominant role for GABAergic pathways in the effects of cannabinoids on hippocampal-dependent memory processes.

Cannabinoid Agonists Impair Hippocampal-Dependent Learning and Memory

In humans, non-human primates, and rodents, cannabinoids impair the performance of a wide variety of memory tasks that share the common feature of requiring the hippocampus for normal performance (Sullivan, 2000; Davies et al., 2000; Kiedel and Davies, 2003). In laboratory rodents, activation of cannabinoid receptors via THC or synthetic analogues such as WIN 55,212-2, CP55940, HU-210 or the endogenous agonist anandamide impairs learning (Davies et al., 2000). Administration of THC disrupts hippocampal-dependent learned behavior in operant and spatial maze models of memory (Nakamura et al., 1991; Heyson et al., 1993; Lichtman et al., 1993; Bradkin and Moerschbacher, 1997; Mallet and Beaulieu, 1998; Ferrari et al., 1999; Vassil et al., 2000). For example, systemic THC administration (2–6 mg/kg i.p.) impairs working memory tested in the radial-arm spatial task and the cannabinoid antagonist SR141716A (1–10 mg/kg) prevents these deficits in a dose-dependent manner (Lichtman and Martin, 1996). Similarly, THC (8 mg/kg) impairs the acquisition of spatial learning in the water maze and the performance of mice in a working memory task, while consolidation and retrieval of a previously learned task are not affected. Pre-treatment with the antagonist SR 141716A (1 mg/kg i.p.) prevents these learning deficits (Do and Galabashi, 2000). Additionally, systemic administration of THC or the synthetic cannabinoid receptor agonist WIN 55,212-2 reliably impairs performance in delayed-match-to-sample and delayed-non-match-to-sample tasks, and this is accompanied by decreases in hippocampal cell firing during the sample phases of the task (Heyson et al., 1993; Hampton and Dendyveler, 1999, 2000).

Overall, the literature discussed above suggests that activation of cannabinoid receptors impairs learning. However, since the agonists were systemically infused, most of these experiments do not specifically show that cannabinoids impair learning and memory via action on the hippocampus. Rather, the involvement of the hippocampus is assumed because it is an important target for systemically administered cannabinoids and because most of the paradigms described are spatial tasks known to be hippocampus-dependent.

More recent research has directly tested whether specific administration of cannabinoids into the hippocampus would have similar effects (summarized in Table 1). Intrahippocampal infusions of the agonists CP55940, THC, or WIN 55,212-2 were found to disrupt performance in the radial-arm maze, and in T-maze delayed alternation, passive avoidance, spatial learning, and place recognition memory tasks (Lichtman et al., 1993; Mishima et al., 2000; Egashira et al., 2002; Suenaga and Ichitani, 2003; Suenaga et al., 2005; Wagoner et al., 2008; Akashi and Mihay, 2010). For example, activation of hippocampal cannabinoid receptors by the agonist WIN 55,212-2 (1–2 µg) dose-dependently decreases the exploration of an object in a new place, and this effect is antagonized by pre-treatment with the cannabinoid receptor antagonist AM 281 (2 mg/kg, i.p.; Suenaga and Ichitani, 2005). WIN 55,212-2 (5 µg)

injected into the dorsal hippocampus increases the number of reference memory errors in the eight-arm radial-maze task, suggesting impairment of memory retrieval (Wiegman et al., 2008). Additionally, post-training intrahippocampal administration of WIN 55,212-2 (2.5 and 5 µg) disrupts long-term spatial memory, but not acquisition or short-term memory, in a rat reference memory task in the water maze (Yim et al., 2008). We have recently found that WIN 55,212-2 administered systemically (0.5 mg/kg) or specifically into the hippocampal CA1 area (5 µg/site) before massed training in the Morris water maze impairs spatial learning (Abuch and Akinyi, 2010). Thus experiments that specifically targeted the hippocampus confirm the implications of the earlier systemic research as to the impairing effect of cannabinoids on hippocampal-dependent learning and memory.

TABLE 1

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Table 1. Effects of intra-dorsal hippocampal WIN 55,212-2 on learning and memory.

Cannabinoid Agonists Impair Hippocampal Synaptic Plasticity

In neuronal circuits, memory storage depends on activity-dependent modifications in synaptic efficacy, such as long-term potentiation (LTP) and long-term depression (LTD), which are the two main forms of synaptic plasticity in the brain. A key feature of LTP and LTD is that a short period of synaptic activity (either high- or low-frequency stimulation) can trigger persistent changes in synaptic transmission lasting at least several hours and often longer. This single property initially led investigators to suggest that these forms of plasticity are the cellular correlate of learning (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). Indeed, efforts to understand synaptic plasticity are driven by the belief that such synaptic modifications might occur during learning and memory. However, it is extremely difficult to demonstrate directly that learning-induced synaptic changes occur following experience.

The mechanisms underlying synaptic plasticity have been studied more intensely in the hippocampus than in any other brain region. Both forms of synaptic plasticity have been studied most intensively at the Schaffer collateral–CA1 synapses of the hippocampus because of the established role of the CA1 area in spatial memory (Behr et al., 2005). LTP and LTD are thought to be involved in memory formation at glutamatergic synapses in the hippocampus. Cannabinoids appear to work by reducing glutamate release below the level needed to activate *N*-Methyl-D-aspartate (NMDA) receptors that are required for LTP and LTD (Shen et al., 1996; Misaner and Sullivan, 1999). CB₁ receptors are capable of regulating both inhibitory and excitatory neurotransmitter release in the hippocampus and are thus capable of exerting subtle control over synaptic plasticity.

Most of our knowledge about cannabinoids and activity-dependent changes in synaptic strength comes from studies performed at excitatory synapses, largely using acute hippocampal slices as the experimental model (Chevalayre et al., 2006). Cannabinoid receptor activation inhibits both LTP and LTD induction in the hippocampal slice. The inhibition of LTP in field potentials in the CA1 region has been demonstrated using THC, HU-210, WIN 55,212-2, 2-AG, and anandamide (Nowicky et al., 1983; Gallus et al., 1994, 1993; Fernandez et al., 1995; Misaner and Sullivan, 1999) and has been found recently to inhibit hippocampal LTD of CA1 field potentials as well (Misaner and Sullivan, 1999). The impairment in the induction of LTP in the CA1 is blocked by cannabinoid antagonists such as SR141716A.

We have recently examined cannabinoid modulation of LTP and LTD in a different experimental model: acute anesthetized rats. Using this experimental condition, we found that i.p. administration of WIN 55,212-2 or the CB₁ receptor antagonist AM251 at the doses tested impairs LTP in the Schaffer collateral–CA1 projection, with no effect on LTD (Abuch and Akinyi, 2010; see Figure 1).

de Oliveira-Almeida et al. (2006) have also demonstrated impairment of LTP in a CA1 slice preparation following AM251 administration. Sakai et al. (2008) found that the CB₁ receptor antagonist SR141716A blocked the potentiation of the fEPSP slope observed following HFS to the perforant path. However, other studies conducted on hippocampal slices of the Schaffer collateral–CA1 synapses have shown that CB₁ blockade favors LTP in the hippocampus (Sharma et al., 2007) and that mice lacking CB₁ receptors show enhanced LTP (Inaba et al., 2000). However, in the study by Sharma et al. (2007), the drug was present throughout the experiment and LTP was elicited by moderate stimulations (20 or 50 pulses). Thus, the discrepancies with our findings could result from the examination of field potential in an intact rat model versus slices, or from various methodological issues, such as different stimulation protocols, different drug doses, etc.

FIGURE 1

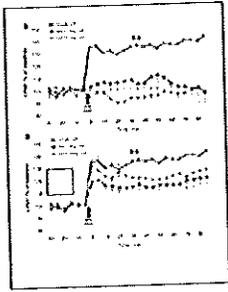


Figure 1. CB₁ receptor antagonist and agonist impair the induction of LTP. (A) AM251 injected i.p. (1 or 2 mg/kg) 30 min before application of high frequency stimulation (HFS; 200 Hz) to the Schaffer collateral significantly impairs the induction of LTP in the CA1 compared with the vehicle group ($P < 0.01$, vehicle differs from all the groups). No significant difference is observed between the groups before HFS. **(B)** WIN 55,212-2 (0.5 mg/kg) injected i.p. 20 min before application of HFS (200 Hz) to the Schaffer collateral significantly impairs the induction of LTP in the CA1 compared with the vehicle group ($P < 0.01$). No significant difference is observed between the groups before HFS. Inset: representative traces in the CA1 for vehicle (upper traces) and WIN 0.5 mg (lower traces) groups taken before (black) and 90 min after (gray) HFS to the Schaffer collateral (calibration: 0.2 mV, 10 μ s). Data published by Mash and Alkay (2010) in *Hippocampus*.

Effects of Cannabinoid Agonists on Emotional and Non-Emotional Memory

Although considerable evidence suggests that activation of CB₁ receptors can induce learning and memory impairments (Sullivan, 2000; Robinson et al., 2009; O'Shea et al., 2003; Varvel et al., 2005), CB₁ receptors are essential for the extinction of conditioned fear associations (Marsicano et al., 2009), indicating an important role for this receptor in neuronal emotional learning and memory.

Role of the Cannabinoid System in Extinction

Extinction was established as a tool to treat conditioned fear by Freud in the 1920s. It has become widely accepted that a deficit in the capacity to extinguish memories of fear is at the root of fear disorders as a result of the distinction between those who do and do not develop serious symptoms after fearsome experiences, and the fact that fear disorders are treated with therapy based on extinction procedures. Moreover, panic attacks, phobias, and particularly post-traumatic stress disorder (PTSD) are viewed by many as a deficit of extinction that should therefore be treated by an intensification of extinction (Charney et al., 1993; Weiss and Flor, 2000; Milad et al., 2008).

Conditioned fear is induced by pairing a neutral, conditioned stimulus (CS; e.g., a light, a tone, or a context) with an aversive stimulus (unconditioned stimulus, US; e.g., a mild footshock) that evokes a measurable fear response. Experimental extinction learning occurs when a CS that previously predicted a US no longer does so, and over time, the conditioned response (e.g., freezing or elevated skin conductance responses) decreases. Extinction learning involves the ventromedial prefrontal cortex (PFC), or amygdala, and hippocampus (Milad and Quirk, 2005; Phelps et al., 2004; Bouton et al., 2006). PTSD patients continue to re-experience the traumatic event over a long timeframe and avoid trauma-related stimuli, even though they recognize that the traumatic event is no longer occurring. It has been suggested that dysfunctional fear extinction plays an important role in the development of clinical symptoms, such as reexperiencing trauma in PTSD (Raibbana and Davis, 2003; Milad et al., 2006; Quirk et al., 2006; Rauch et al., 2006). PTSD patients also demonstrate impaired extinction in the aftermath of new trauma. For example, Milad et al. (2008) have shown deficient extinction recall as measured in skin conductance response in a 2-day fear conditioning and extinction procedure in PTSD patients.

Clearly, animal models do not entirely mimic the complex features of psychiatric disorders. However, they can predict the clinical effects of substances and provide insights into the biological mechanisms of these diseases. Marsicano et al. (2009) found that CB₁ receptor-deficient mice show normal acquisition and consolidation in a fear conditioning task, but fear extinction is strongly impaired. Impaired extinction is also observed when the antagonist SR141716 is injected systemically into wild-type mice before the extinction trial, indicating that CB₁ receptors are required at the moment of the extinction training. The findings that CB₁ knockout mice exhibit impaired short- and long-term extinction of cue-induced conditioned fear responses have been replicated by other groups for the extinction of both cue- and context-induced fear responses (Finn et al., 2003; Suzuki et al., 2004; Chidambaram et al., 2005; Lafenêtre et al., 2007; Lutz, 2007; Nishitani et al., 2007). We have recently shown that microinjecting the antagonist AM251 (6 ng) into the BLA or the CA1 significantly impairs extinction of inhibitory avoidance (Gambus-Blazyn and Alkay, 2009; Mash and Alkay, 2010). Several studies suggest that the eCB system is not involved in the extinction of non-aversive memories (Hilber et al.,

On the other hand, studies have demonstrated that pharmacological activation of eCB signaling promotes extinction of fear memories. For example, Chhatwal et al. (2003) found that systemic administration of the eCB transporter AM404 (10 mg/kg) promotes extinction of fear that was conditioned using fear-potentiated startle. This was replicated using systemic (Pamplona et al., 2008) and intracerebroventricular (Bibiak et al., 2008) injections. In another study (Navvel et al., 2007), OL-135 (30 mg/kg), an inhibitor of FAAH, enhanced the rate of extinction in a water maze task. Pamplona et al. (2006) showed that WIN 55,212-2 (0.25 mg/kg) facilitates the extinction of contextual fear in the fear conditioning task and of spatial memory in the water maze reversal task. We have used the light–dark inhibitory avoidance procedure to demonstrate the effects of WIN 55,212-2 administered into the CA1 or the BLA on extinction. This procedure is dependent on both the amygdala and hippocampus as a single CS–US (context–footshock) pairing establishes a robust long-term memory, expressed as an increase in latency to enter the dark chamber at testing. Repeated retrieval of the avoidance response in the absence of the US induces extinction of inhibitory avoidance memory, meaning that the animal learns that the context no longer predicts the footshock. We found that WIN 55,212-2 administered into the CA1 facilitates the extinction of inhibitory avoidance, with no effect on extinction kinetics when microinjected into the BLA (Gannon-Elazar and Akirav, 2009; Abush and Akirav, 2010).

Hence, the results of Marsicano et al. (2002) and subsequent investigations demonstrate that inhibition of eCB transmission robustly inhibits (or prolongs) fear extinction (Suzuki et al., 2004; Pamplona et al., 2006; Gannon-Elazar and Akirav, 2009; Abush and Akirav, 2010). Conversely, stimulation of eCB transmission accelerates fear extinction (Suzuki et al., 2004; Chhatwal et al., 2007; Barad et al., 2006; Abush and Akirav, 2010).

Comparing the Effects of Cannabinoid Agonists on Aversive and Non-Aversive Tasks

It has been suggested that the neural processes underlying emotional memory formation (such as extinction learning) and non-emotional memories (such as spatial learning) are differentially sensitive to cannabinoid receptor activation (Chhatwal and Resniko, 2007). An intriguing question is whether cannabinoids have a similar effect on other types of emotional memories that do not involve fear and extinction learning.

We have recent findings suggesting that cannabinoid receptor activation has differential effects on learning and memory that are task-, brain region-, and memory stage-dependent (Seger and Akirav, 2011). We examined the effects of WIN 55,212-2 microinjected into the amygdala and the subiculum on the acquisition and retrieval of a neutral learning task (i.e., social discrimination) and an aversive learning task (i.e., contextual fear conditioning). The subiculum is the principal target of CA1 pyramidal cells. It functions as a mediator of hippocampal–cortical interaction and has been proposed to play an important role in the encoding and retrieval of long-term memory. In fear conditioning paradigms, the BLA plays a central role in the formation and consolidation of fear-related memory traces (LeDoux, 2003; Maren and Clark, 2001), whereas the hippocampus's role is to integrate the features of the context and not to form a context–shock association (Fanselow, 1988). Unlike the aversive fear conditioning task, social discrimination is considered neutral or even rewarding. This finding was established using both conditioned place preference paradigms and T-maze learning rewarded by social interaction (Yanakei-Hara et al., 1999). Social recognition processes depend on brain regions such as the medial amygdala, which modulates the initial social encounter and formation of social memory (Ferguson et al., 2000; Belsky and Young, 2004) and the ventral hippocampus (Van Wimersma Greidanus and Majgor, 1996; Kogan et al., 2000).

We found that in the aversive contextual fear task, WIN 55,212-2 administered into the BLA impairs fear acquisition/consolidation, but not retrieval, whereas in the ventral subiculum (vSub), WIN 55,212-2 impairs fear retrieval. In the non-aversive or rewarding social discrimination task, WIN 55,212-2 into the vSub impairs acquisition/consolidation and retrieval, whereas in the medial amygdala, WIN 55,212-2 impairs acquisition (Seger and Akirav, 2011). These findings suggest that cannabinoid agonists can impair emotional (or aversive) as well as neutral (or rewarding) memory-related processes in a task-, region-, and memory stage-dependent manner. This is consistent with other studies suggesting that exogenous acute cannabinoid treatment may have different outcomes depending on task aversiveness and the brain region involved (Suzuki et al., 2004; de Oliveira Alvares et al., 2006; Navvel et al., 2007; Gannon-Elazar and Akirav, 2009; Abush and Akirav, 2010).

Effects of Cannabinoids on Stress and Anxiety

Considerable evidence suggests that cannabinoids are anxiolytics and modulate the behavioral and physiological response to stressful events (Vivavos et al., 2007; Hill et al., 2001). Consequently, the effects of CB₁ agonists on learning and memory may be attributable to a general modulation of anxiety or stress levels and not to memory *per se*.

Stress is most readily defined as any stimulus that presents a challenge to homeostasis including any actual or potential disturbance

of an individual's environment. The stress response enables the animal to adapt to the changing environment (Joels and Lupino, 2000). Fear is an adaptive component of the acute stress response to potentially dangerous stimuli that threaten the integrity of the individual. However, when disproportionate in its intensity, chronic, irreversible, and/or not associated with any actual risk, it constitutes a maladaptive response and may be symptomatic of anxiety-related neuropsychiatric disorders (Cabe and Huber, 2000).

Anxiety disorders are marked by excessive fear (and avoidance), often in response to specific objects or situations, in the absence of true danger, and they are common in the general population (Shin and Liberzon, 2010). As excessive fear is a key component of anxiety disorders, the search for the neurocircuitry of anxiety disorders has focused extensively on studies of fear circuits in animal models. These studies examined the neurocircuitry associated with fear responses in rats and mice using fear conditioning paradigms, inhibitory avoidance, and fear-potentiated startle models. The amygdala, PFC, and hippocampus have arisen as clear regions of interest in studies of anxiety disorders and are implicated in PTSD (Shin and Liberzon, 2010).

The hippocampus is often implicated in the neurobiology of stress. Mineralocorticoid and glucocorticoid receptors are expressed in high numbers within the hippocampus. Although stress-induced corticosteroid signaling in the hippocampus has a beneficial role in regulating the time course of the hypothalamic–pituitary–adrenal (HPA) axis stress response (De Kloet et al., 2005), prolonged glucocorticoid signaling can damage the hippocampus as measured by dendritic atrophy, decreased neurogenesis, and deficits in synaptic plasticity (McEwen and Gould, 1990; Sapolsky, 1996; McEwen, 1999; Meaney, 2001). In PTSD and major depression patients, hippocampus volumes are reduced (Bremner et al., 1993; Sheline et al., 1999; Woon and Hodges, 2008), and smaller hippocampal volumes are predictive of vulnerability to developing stress-related disorders (Pitman et al., 2009).

Role of the Endocannabinoid System in Unconditioned Stress and Anxiety

Results from many studies indicate that the eCB system modulates unconditioned stress- and anxiety-like responses (Viveros et al., 2003; Gorzalka et al., 2008; Lutz, 2009). A general conclusion that can be tentatively derived from the complicated and often contradictory literature is that inhibition of eCB signaling increases stress and anxiety, while moderate increases in eCB signaling decrease stress and anxiety (Lutz, 2009; summarized in Table 2). The term “moderate” is used because strong stimulation of eCB signaling by high doses of CB₁ receptor agonists potentiates stress- and anxiety-like responses (Rodriguez de Fonseca et al., 1996; Scherma et al., 2003; Lutz, 2009). This biphasic effect has been demonstrated in animal models of anxiety (Lafenêtre et al., 2007; Hill and Gorzalka, 2009), and also in humans. Cannabis may induce aversive states in some smokers, precipitating anxiety and panic attacks (Hill and Solowij, 1998). Furthermore, THC administration may result in psychotic-like states (Eisen and van Amelsvoort, 2007). These bidirectional effects of cannabinoids observed in humans can be mimicked in laboratory animals. Hence, in models predictive of anxiolytic-like activity, low doses of CB₁ agonists tend to be anxiolytic and high doses tend to increase aversion and anxiety-related behaviors (Viveros et al., 2005).

TABLE 2

Table 2. Effects of cannabinoids on anxiety-related responses.

Procedures used in studies on the role of eCBs in stress and anxiety evaluate the anxiolytic/anxiogenic effects of drugs by using standard tasks such as the elevated plus maze (EPM), social interaction, and defensive burying (Viveros et al., 2005; Lutz, 2009). Using the EPM, Patel and Hillard (2006) found that cannabinoid receptor agonists WIN 55212-2 (0.3–10 mg/kg) and CP55940 (0.001–0.3 mg/kg) administered systemically increase the time mice spend on the open arms (i.e., elicit an anxiolytic response) only at low doses. At the highest doses, both compounds alter overall locomotor activity. In contrast, THC (0.25–10 mg/kg) produces a dose-dependent reduction in time spent on open arms. The eCB uptake/catabolism inhibitor AM404 (0.3–10 mg/kg) produces an increase in time spent on the open arms at low doses and has no effect at the highest dose tested. The FAAH inhibitor URB597 (0.03–0.3 mg/kg) produces a monophasic, dose-dependent increase in time spent on the open arms. Systemic administration of the CB₁ receptor antagonists SR141716 (1–10 mg/kg) and AM251 (1–10 mg/kg) produce dose-related decreases in time spent on open arms. Onuki et al. (1999) have shown that THC induces increased aversion to the open arms of the EPM in both rats and mice that is similar to the aversion produced by anxiogenic agents. In contrast, mice treated with the agonists cannabidiol and nabilone spend a greater amount of time in the open arms of the maze, an effect similar to that produced by diazepam, the reference anxiolytic agent.

In the light–dark box, Bernocchi and Maldonado (2007) have shown that the systemic administration of a low dose of THC (0.3

mg/kg) produces clear anxiolytic-like responses. The CB₁ cannabinoid receptor antagonist SR 141716A (0.5 mg/kg) completely blocks the anxiolytic-like response induced by THC, suggesting that this effect is mediated by CB₁ cannabinoid receptors. In another study, systemic administration of the FAAH inhibitors URB597 and URB532 reduces anxiety-related behavior in the rat elevated zero-maze and in isolation-induced ultrasonic vocalization tests (Kathuria et al., 2009). These effects are dose-dependent and blocked by the antagonist rimonabant. The FAAH inhibitor and eCB re-uptake inhibitor AM404 also exhibit a dose-dependent anxiolytic profile in the EPM, defensive withdrawal test, and ultrasonic vocalization test (Bartalucci et al., 2006). URB597 has also been shown to be anxiolytic in the rat EPM and open-field tests (Hill et al., 2007) and has recently been shown to reduce anxiety-related behavior in the EPM in Syrian hamsters (Majid et al., 2008).

Ribbaño et al. (2009) examined the dose-response effects of exogenous anandamide at doses of 0.01, 0.1, and 1.0 mg/kg in mice sequentially submitted to the open field and EPM. Systemically administered at 0.1 mg/kg (but not at 0.01 or 1 mg/kg), anandamide increases the time spent and the distance covered in the central zone of the open field, as well as exploration of the open arms of the EPM. Recently, Ribbaño et al. (2009b) demonstrated that the anxiolytic-like effect of a low anandamide dose is reversed by administration of the antagonist AM251, whereas the anxiogenic-like effect is inhibited by pre-treatment with capsazepine, a transient receptor potential vanilloid type 1 (TRPV1) receptor antagonist. The authors suggested that the anxiolytic effect evoked by anandamide might be due to the interaction with the CB₁ cannabinoid receptor, whereas vanilloid receptors seem to be involved in the anxiogenic action of anandamide (Ribbaño et al., 2009b). Marsh et al. (2007) reported that TRPV1 “null” mice exhibit a significantly reduced response to anxiogenic stimuli. Therefore, the anandamide-induced inverted U-shape pattern might be based on the fact that the intrinsic efficacy of anandamide on TRPV1 is relatively low compared to that observed on the CB₁ receptor (Ross, 2007).

Transgenic mice deficient for FAAH, the enzyme that degrades anandamide, demonstrate reduced anxiety-like behavior in the EPM and light–dark box compared with wild-type mice and these effects are prevented by systemic administration of the antagonist rimonabant (Maremón et al., 2008). By contrast, transgenic mice lacking expression of the CB₁ receptor demonstrate an anxiogenic profile in the EPM, the light–dark box, open-field arena, and social interaction test (Haller et al., 2002, 2003; Maccagnano et al., 2002; Marín et al., 2003; Urigien et al., 2003) and demonstrate impaired stress coping behavior in the forced swim test (Steiner et al., 2008). Similarly, CB₁ receptor antagonists increase anxiety-related behaviors in the EPM (Paisi and Holland, 2006). Taken together, these studies suggest that eCBs act at CB₁ receptors to reduce anxiety.

Role of the Endocannabinoid System in Conditioned Fear and Anxiety

Understanding the role of the eCB system in conditioned fear and aversive memories is important because a number of anxiety disorders, including PTSD and phobias, are thought to result from dysregulated fear neurocircuitry (Bañez et al., 2006). Investigators have examined the effect of CB₁ receptor agonists and antagonists on contextual and cue fear conditioning. Results from these studies were somewhat mixed. In rats, systemic injections of the CB₁ receptor antagonist AM251 enhance both the acquisition and expression of cue fear conditioning (Vannas et al., 2006; Rebec et al., 2008). Administering AM251 (5 mg/kg, i.p) during tone–footshock conditioning enhances acquisition of freezing behavior for both trace fear conditioning (hippocampal-dependent) and delay fear conditioning (amygdala-dependent; Rebec et al., 2008). Recently, we used an inhibitory avoidance task and found that microinjecting AM251 (6 ng) into the BLA significantly enhances conditioned avoidance but has no effect on conditioning when microinjected into the hippocampal CA1 area (Gannon-Plazar and Akirav, 2009; Akirav and Akirav, 2010). However, others have shown that mice lacking the CB₁ receptor or systemically administered with the CB₁ receptor antagonist AM251 (0.3–3 mg/kg) 30 min before behavioral testing show no contextually induced fear response (Mebius et al., 2006). Furthermore, the CB₁ receptor antagonist rimonabant or genetic deletion of the CB₁ receptor has no effect on the acquisition of cue and context fear conditioning in mice (Mao-Jinn et al., 2009; Suzuki et al., 2003). On the other hand, cue-fear-potentiated startle is decreased by medial PFC injections of the CB₁ receptor agonist WIN 55212-2 or the FAAH inhibitor URB597 (Li et al., 2008, 2009) and contextual fear conditioning is decreased by dorsolateral periaqueductal gray injections of either anandamide or the anandamide transport inhibitor AM404 (Bossier et al., 2008). Overall it appears that, as in the case of unconditioned fear, inhibition of eCB transmission increases fear while moderate stimulation of eCB transmission decreases fear.

The Involvement of the Hippocampus in Endocannabinoid Modulation of Stress and Anxiety

Techniques based on intracranial injections of cannabinoids in rats revealed that activation of CB₁ receptors is involved in inducing anxiolytic- or antidepressant-like effects (Bañez et al., 2007; Maccagnano et al., 2003; Ribbaño et al., 2003a,b). For example, Ribbaño et al. (2003a) found that low doses of THC microinjected into the PFC (10 µg) or ventral hippocampus (5 µg) in rats induces an

anxiolytic-like response during tests in the EPM, while higher doses do not show an anxiolytic effect and even seem to switch into an anxiogenic profile. Nevertheless, other studies demonstrated that eCB activation in the amygdala and dorsal hippocampus results in an anxiogenic-like response. Low THC doses (1 μg) in the BLA produce an anxiogenic-like response whereas higher doses are ineffective (Rubino et al., 2008a). WIN-55212-2 in the dorsal hippocampus (2.5 and 5 μg) produces a significant anxiogenic-like effect in rats that is reversed by AM251 (Rothblat et al., 2007).

Local infusion of cannabinoid compounds into specific brain areas might be instrumental in identifying neural pathways and neuroanatomically separated CB₁ receptor subpopulations that may play distinct roles in and mediate the opposing actions of cannabinoids, notably, anxiolytic versus anxiogenic effects (Marelli et al., 2009; Viveros et al., 2007). We examined the role of cannabinoids in modulating aversive and non-aversive learning paradigms in the hippocampus and amygdala (Grueter-Blazar and Akirav, 2009; Abush and Akirav, 2010; Segov and Akirav, 2011). Microinjecting the antagonist AM251 (6 ng) or the agonist WIN-55212-2 (5 μg) into the BLA, CA1, or vSub had no effect on anxiety levels as measured in the open-field, pain sensitivity (Grueter-Blazar and Akirav, 2009; Abush and Akirav, 2010; Segov and Akirav, 2011), or EPM tests (Abush and Akirav, 2010). However, both agonist and antagonist had profound effects on aversive and non-aversive learning tasks. These findings suggest that in these studies the impairing and facilitating effects of local infusions of WIN-55212-2 on learning and memory are probably not attributable to a general modulation of anxiety. Nevertheless, the effects of cannabinoids on the interplay between anxiety and memory processes are difficult to separate and further examination of the effects of different cannabinoids is required.

To summarize the role of the eCB system in stress, anxiety, and conditioned fear, there is a general consensus that the effects of cannabinoid agonists on anxiety seem to be biphasic, with low doses being anxiolytic and high doses being ineffective or possibly anxiogenic. There are several important characteristics of the eCB system that might explain these different effects of eCB modulation. First, in a physiological situation, eCB synthesis, and thus CB₁ receptor activation, occurs in particular activated neuronal circuits. This is a notable difference from the situation following pharmacological treatment with receptor agonists, when the agent activates all CB₁ receptors in the brain regardless of their specific involvement in a particular physiological process. Second, the CB₁ receptor is expressed in diverse brain structures of relevance to psychiatric disorders and is mainly located presynaptically where it can suppress the release of other neurotransmitters (Mansimone and Lutz, 1999, 2005; Mackie, 2007). These neurotransmitters include the main inhibitory neurotransmitter GABA, the main excitatory neurotransmitter glutamate, as well as acetylcholine, noradrenaline, and serotonin (Katona et al., 1999; Harkany et al., 2003; Mooney et al., 2000; Häring et al., 2007; Oropeza et al., 2011). Thus, synthetic compounds delivered systemically lack both the spatial and temporal specificity of endogenous compounds (Lafenêtre et al., 2007; Viveros et al., 2007; Moreira and Lutz, 2008). This may explain not only the bell-shaped relationship between dose and effect that some studies have observed, but also why elevation of eCB levels sometimes has effects that are different from those observed with exogenous cannabinoids. Finally, the diversity of eCB ligands with their multiple synthetic and degradation pathways adds a further level of complexity to the eCB system (Di Marzo, 2008).

Summary

The findings demonstrate that the cannabinoid system has diverse effects on hippocampal memory and plasticity that cannot be categorized simply into an impairing or an enhancing effect, but are rather dependent on important variables such as the nature of the task (i.e., aversive, emotional or not), the memory stage under investigation (acquisition, consolidation, retrieval, extinction), and the brain areas involved.

The involvement of the eCB system in multiple aspects of brain function provides new targets for the development of novel therapeutic agents for a wide range of psychiatric disorders, including the treatment of anxiety disorders. Studies examining the involvement of cannabinoids in memory processes advance our understanding of the potential harmful consequences of cannabis use and the mechanisms underlying the close relationship between cannabinoids and cognition. This will help in determining whether the clinical benefits of using cannabinoids outweigh the risks, and to better cope with the deficits induced by cannabinoids.

Conflict of Interest Statement

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Exhibit B

STUDY

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

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2009

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 venlafaxine, 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 (CB₁ and CB₂), possibly one or more atypical receptors, and several ligands (notably anandamide and 2-arachidonolglycerol [2-A]). The CB₁ re-

ceptor is distributed primarily within the CNS, particularly in the cerebellum, basal ganglia, amygdala, cerebral cortex, and hippocampus [12,13]. The CB₂ 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 (CB₁ and CB₂) 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 \pm SD	44 \pm 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 night sweats while on nabilone. Once effective relief of nightmares was achieved, no

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Exhibit C

STUDY

*The Endogenous Cannabinoid System
Controls Extinction of Aversive
Memories*

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August, 2002

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Competing interests statement

The authors declare that they have no competing financial interests.

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The endogenous cannabinoid system controls extinction of aversive memories

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Acquisition and storage of aversive memories is one of the basic principles of central nervous systems throughout the animal kingdom¹. In the absence of reinforcement, the resulting behavioural response will gradually diminish to be finally extinct. Despite the importance of extinction², its cellular mechanisms are largely unknown. The cannabinoid receptor 1 (CB1)³ and endocannabinoids⁴ are present in memory-related brain areas^{5,6} and modulate memory^{7,8}. Here we show that the endogenous cannabinoid system has a central function in extinction of aversive memories. CB1-deficient mice showed strongly impaired short-term and long-term extinction in auditory fear-conditioning tests, with unaffected memory acquisition and consolidation.

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Treatment of wild-type mice with the CB1 antagonist SR141716A mimicked the phenotype of CB1-deficient mice, revealing that CB1 is required at the moment of memory extinction. Consistently, tone presentation during extinction trials resulted in elevated levels of endocannabinoids in the basolateral amygdala complex, a region known to control extinction of aversive memories⁹. In the basolateral amygdala, endocannabinoids and CB1 were crucially involved in long-term depression of GABA (γ -aminobutyric acid)-mediated inhibitory currents. We propose that endocannabinoids facilitate extinction of aversive memories through their selective inhibitory effects on local inhibitory networks in the amygdala.

To study the involvement of the endogenous cannabinoid system in memory processing, we generated CB1-deficient mice (*CB1*^{-/-}; see Supplementary Information). *CB1*^{-/-} mice and *CB1*^{+/+} littermates were tested in auditory fear conditioning, which is highly dependent on the amygdala¹ and enables the dissection of different phases of memory formation, including acquisition, consolidation and extinction. Mice were trained to associate a tone with a foot-shock (conditioning). After conditioning, animals froze when

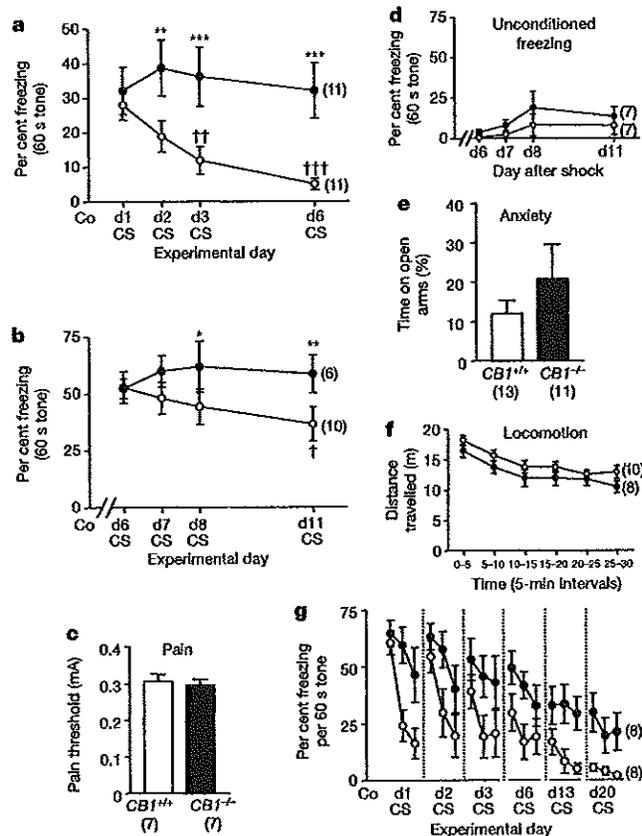


Figure 1 Impaired extinction of aversive memory in an auditory fear-conditioning task of *CB1*^{-/-} mice (filled circles) as compared to their *CB1*^{+/+} littermates (open circles). **a**, **b**, After conditioning (Co) animals were repeatedly exposed to 60 s tones (conditioned stimulus, CS) starting 24 h after conditioning (**a**) (d1) or after a 6-day consolidation period (**b**) (d6). **c–f**, *CB1*^{-/-} and *CB1*^{+/+} mice did not differ in their sensory-motor abilities, as assessed by sensitivity to rising electric foot-shock (**c**), unspecific freezing to a tone after shock application (**d**), anxiety-related behaviour on the elevated plus maze (**e**) and horizontal locomotion in an open field (**f**). **g**, *CB1*^{-/-} mice showed memory extinction in response to a stronger extinction protocol (3 min tones until day 20; analysed in 60-s intervals), but still froze more than *CB1*^{+/+} controls. Means \pm s.e.m. are shown; number of animals are indicated in parentheses. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$ (compared with *CB1*^{+/+}); dagger, $P < 0.05$; double dagger, $P < 0.01$; triple dagger, $P < 0.001$ (compared with day 1).

re-exposed to the tone. This response served as an indicator of aversive memory, and is gradually extinguished on repeated tone presentations. As the amygdala has a crucial role for extinction of aversive memories^{9,10}, we studied amygdala-dependent memory performance in the absence of possible confounding influences of the hippocampus by re-exposing the mice to the tone in an environment different from the conditioning context¹. In this environment, neither *CBI*^{-/-} nor *CBI*^{+/+} mice showed freezing without tone presentation 24 h after conditioning (data not shown). During the subsequent tone presentation, however, animals of both groups showed the same amount of freezing (Fig. 1a; d1, $P > 0.05$), pointing to an equally successful tone-foot-shock association. On repeated exposure to the tone, however, *CBI*^{+/+} and *CBI*^{-/-} mice differed significantly in their freezing behaviour (genotype: $F_{1,20} = 5.81$, $P < 0.05$; genotype \times day interaction: $F_{3,60} = 4.86$, $P < 0.005$; Fig. 1a). In fact, *CBI*^{+/+} mice ($F_{3,10} = 9.70$, $P < 0.0005$), but not *CBI*^{-/-} ($F_{3,10} = 0.94$, $P = 0.433$), showed extinction of freezing.

The identical behavioural performance of the two genotypes on day 1 indicates that acquisition and early consolidation processes do not involve CB1. However, it is possible that memory consolidation processes were not completed 24 h after conditioning, leaving open a potential involvement of CB1 in later phases of memory consolidation. To test this hypothesis, new groups of animals remained undisturbed after conditioning for 6 days, and mice from these groups were then exposed to the 60-s tones (Fig. 1b). Again, *CBI*^{-/-} and *CBI*^{+/+} mice did not differ in their initial freezing response, but behaved in a significantly different way in the course of repeated tone presentations (genotype \times day interaction: $F_{3,42} = 3.03$, $P < 0.05$). Whereas *CBI*^{+/+} mice showed a decrease in freezing behaviour until day 11 ($F_{3,27} = 3.73$, $P < 0.05$), *CBI*^{-/-} mice failed to extinguish the freezing response ($F_{3,15} = 1.03$, $P = 0.404$). A more detailed analysis of the freezing response in 20-s intervals confirmed the difference in extinction (genotype \times 20-s bin interaction: $F_{11,154} = 2.60$, $P < 0.005$; Supplementary Information). These differences were due to altered short-term and long-term extinction in *CBI*^{-/-} mice but not to increased spontaneous recovery of the freezing response (genotype:

$F_{1,14} = 0.18$, $P = 0.675$; genotype \times day interaction: $F_{2,28} = 1.61$, $P = 0.217$; Supplementary Information).

We next analysed whether the differences in memory extinction between the two genotypes could be attributed to alterations in sensory-motor abilities of *CBI*^{-/-} mice, as cannabinoids are known to influence pain perception, emotionality and locomotion^{4,11,12}. However, mice of either genotype showed the same pain sensitivity to a rising electric foot-shock defined as the shock intensity at which mice showed first signs of discomfort, that is, jumping and/or vocalization (Fig. 1c). Moreover, if the same animals were repeatedly exposed to the tone, there were no significant differences in freezing behaviour between the genotypes (genotype: $F_{1,12} = 1.61$, $P = 0.228$; genotype \times day interaction: $F_{3,36} = 0.225$, $P = 0.878$; Fig. 1d), indicating that CB1 deficiency does not affect foot-shock-induced behavioural sensitization or unconditioned freezing to the tone. Anxiety-related behaviour was analysed on an elevated plus maze. Animals of either genotype spent the same relative time on open arms of the maze ($P > 0.05$, t -test and U -test; Fig. 1e), and made the same relative number of entries into open arms (*CBI*^{+/+}: $22.0 \pm 4.0\%$; *CBI*^{-/-}: $21.1 \pm 7.6\%$, $P > 0.05$, t -test and U -test). In contrast, *CBI*^{-/-} mice showed reduced exploratory activity (number of closed-arm entries: 11.6 ± 1.1 in *CBI*^{+/+} mice compared with 6.5 ± 1.2 in *CBI*^{-/-} mice, $P < 0.01$, t -test). However, in an open-field locomotor activity test, no significant differences were found, including horizontal (Fig. 1f) and vertical locomotion, resting time, and time spent close to the walls of the box (data not shown).

The failure of *CBI*^{-/-} mice to diminish their freezing response during a limited number of 60-s tone presentations (Fig. 1a, b) raises the question as to whether *CBI*^{-/-} mice are able to extinguish aversive memories at all. Thus, conditioned *CBI*^{-/-} and *CBI*^{+/+} mice were exposed to a stronger extinction protocol (3 min tone, six exposures; Fig. 1g). Both *CBI*^{+/+} ($F_{17,119} = 15.01$, $P < 0.000001$) and *CBI*^{-/-} mice ($F_{17,119} = 7.59$, $P < 0.000001$) extinguished their freezing response over the course of repeated tone presentations. Nevertheless, extinction was still more pronounced in *CBI*^{+/+} as compared with *CBI*^{-/-} mice (genotype: $F_{1,14} = 5.30$, $P < 0.05$). Notably, the most marked differences between *CBI*^{-/-}

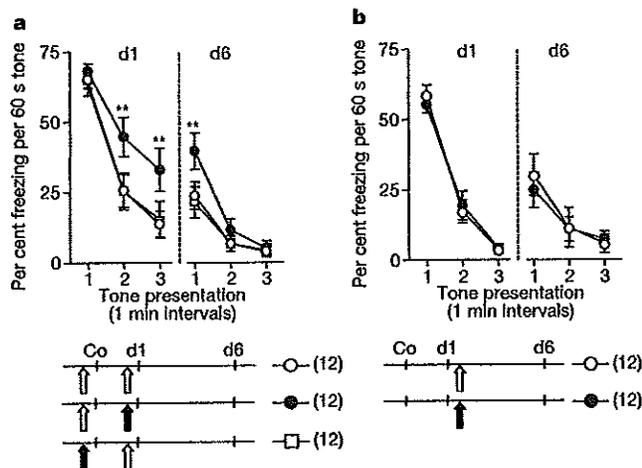


Figure 2 CB1 antagonist SR141716A impairs short-term and long-term extinction, but not acquisition and consolidation of aversive memories. **a**, Mice were treated with SR141716A (filled arrows) or vehicle (open arrows) 20 min before conditioning (Co) and the first extinction trial (d1; 3 min tone). **b**, Mice were treated with SR141716A or vehicle 10 min after the first extinction trial, as indicated. Freezing was analysed in 60-s intervals. Means \pm s.e.m. are shown; number of animals are shown in parentheses. Double asterisk, $P < 0.01$ (compared with the two other groups).

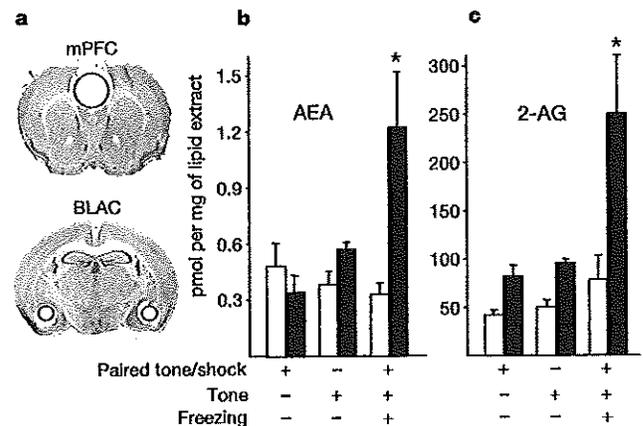


Figure 3 Re-exposure to the tone 24 h after conditioning causes increased endocannabinoid levels in the basolateral amygdala complex (BLAC) but not the medial prefrontal cortex (mPFC) of C57BL/6J mice. **a**, Micrographs of coronal brain sections showing representative examples of the dissected mPFC and BLAC. Circles indicate the size and positioning of tissue sampling. **b**, **c**, Anandamide (**b**, AEA) and 2-arachidonoylglycerol (**c**, 2-AG) levels of the three experimental groups (see text), which differed in conditioning procedure, re-exposure to the tone and resulting freezing response to the tone. Means \pm s.e.m. are shown ($n = 4$ per group, 5 mice per n). Open bars, mPFC; filled bars, BLAC. Asterisk, $P < 0.05$ (compared with BLAC of the other groups).

letters to nature

and $CBI^{+/+}$ mice were observed during acute tone presentation (short-term extinction). Therefore, $CBI^{-/-}$ mice might be primarily impaired in short-term extinction, with a resulting impairment in long-term extinction, assessed in the course of the subsequent extinction trials. Accordingly, spontaneous recovery was not different between the genotypes (genotype: $F_{1,14} = 1.73$, $P = 0.208$; genotype \times day interaction: $F_{4,56} = 1.19$, $P = 0.323$; Supplementary Information).

Our behavioural data clearly indicate an involvement of the endogenous cannabinoid system in extinction of aversive memories. However, the life-long absence of CB1 could result in developmental defects leading to the phenotype observed. It, furthermore, precludes any temporal dissection of the involvement of the endogenous cannabinoid system in different stages of memory formation. Thus, we treated wild-type C57BL/6J mice with the CB1 antagonist SR141716A (ref. 13), either before conditioning, or before the first extinction trial. Systemic application of SR141716A 20 min before the first extinction trial impaired both short-term and

long-term extinction of the freezing response as compared with both vehicle-treated controls and animals treated with SR141716A before conditioning (treatment \times time interaction: $F_{10,160} = 2.72$, $P < 0.005$), with no difference between the two latter treatments and with a similar performance of all three groups in the beginning of the first extinction trial (Fig. 2a). These data largely confirm the phenotype of $CBI^{-/-}$ mice (Fig. 1a, b, g), indicating that endocannabinoids have only a negligible function in memory acquisition, consolidation and recall (indicated by the similar performance at the beginning of the first extinction trial), but selectively interfere with extinction of the freezing response to the tone. Mice treated with SR141716A before the first extinction trial showed an attenuated extinction of freezing not only during the first tone presentation (short-term extinction) but also in the absence of pharmacological treatment during the first 60 s of tone presentation at day 6 (long-term extinction). Spontaneous recovery of the behavioural performance from the end of the first (day 1) to the beginning of the second tone presentation session (day 6) was not different among the three groups ($F_{2,34} = 0.29$, $P = 0.744$; Supplementary Information). Together, these findings support the idea that CB1 might be particularly important for the extinction of acute responses to the tone (short-term extinction), which, in turn, relates to behavioural extinction over repeated tone presentations (long-term extinction), without affecting spontaneous recovery of the behavioural performance. Accordingly, the CB1 antagonist had to be present at the time of tone presentation (that is, during aversive memory recall) in order to interfere with memory extinction, as SR141716A failed to affect extinction if administered immediately at the end of the extinction trial (data not shown) or 10 min later (Fig. 2b).

These observations, together with the pharmacokinetics of SR141716A (ref. 14), led us to assume that presentation of the tone during the extinction trial causes an instantaneous rise in endocannabinoid levels. To confirm this assumption, we measured in C57BL/6J mice levels of the two major endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), in brain punches of the medial prefrontal cortex (mPFC) and the basolateral amygdala complex (BLAC), both of which are thought to have central roles in extinction of aversive memories^{9,15}. In those animals forming an association between tone and foot-shock, levels of AEA and 2-AG were significantly higher in the BLAC at the end of tone presentation of the extinction trial on day 1, as compared with animals with unpaired tone and foot-shock presentation on the previous day and with animals with paired tone and foot-shock presentation but no re-exposure to the tone (Fig. 3). There were no significant differences in levels of AEA and 2-AG in the mPFC, suggesting a specific involvement of endocannabinoids in extinction processes within the BLAC. Data of the two control groups indicate that both a successful tone-foot-shock association and re-exposure to the tone are required to trigger the acute increase of endocannabinoid levels.

If the endogenous cannabinoid system is activated during tone presentation, how exactly does it facilitate memory extinction? To answer this question, we performed a series of electrophysiological experiments in the BLAC of brain slices from $CBI^{-/-}$ and $CBI^{+/+}$ mice. Basic electrical properties were similar in $CBI^{-/-}$ and $CBI^{+/+}$ littermates, including input resistance and resting membrane potential (data not shown). High-frequency stimulation (HFS) in the lateral amygdala close to the external capsule induced long-term potentiation (LTP) in the basolateral amygdala of both genotypes (Fig. 4a). This effect was significantly more pronounced in $CBI^{-/-}$ than in $CBI^{+/+}$ mice (potentiation of population spike amplitude to $147 \pm 11\%$ in $CBI^{-/-}$ compared with $117 \pm 8\%$ in $CBI^{+/+}$ mice, $n = 9$, $P < 0.05$). However, we failed to affect basal synaptic transmission and LTP induction in wild-type slices superfused with SR141716A (5 μ M; data not shown). This indicates that the enhanced LTP in $CBI^{-/-}$ mice might reflect long-term develop-

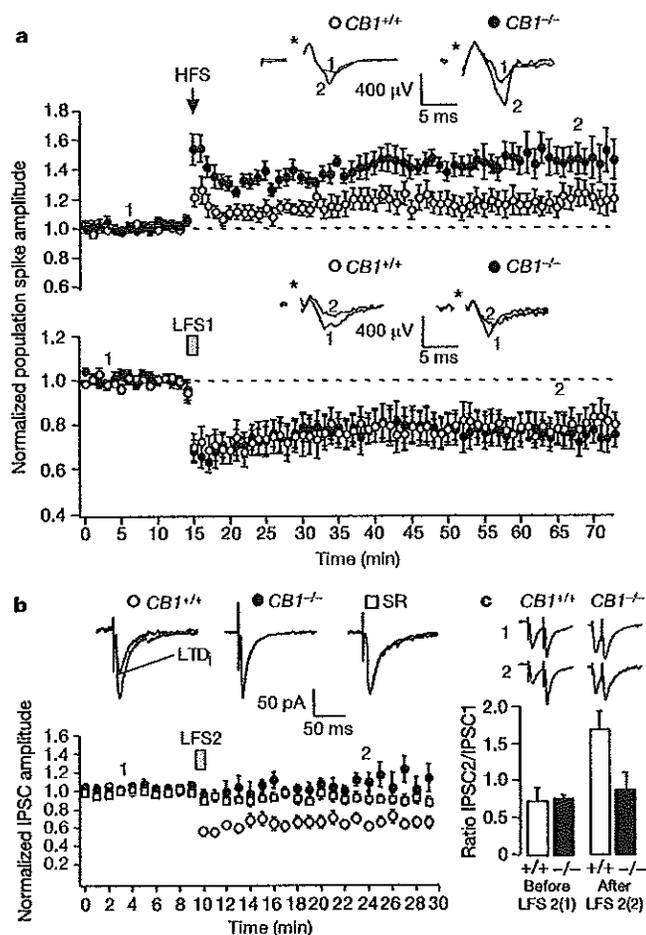


Figure 4 Endogenous cannabinoid system and synaptic plasticity in the basolateral amygdala. **a**, LTP (top) and LTD (bottom) in slices from $CBI^{+/+}$ and $CBI^{-/-}$ mice, induced by high-frequency stimulation (HFS) and low-frequency stimulation (LFS 1), respectively. Asterisks indicate stimulus artefacts. **b**, Long-term depression of IPSCs (LTD) requires CB1 activation. In principal neurons of slices of $CBI^{+/+}$ mice, low-frequency stimulation (LFS 2) induced a reduction of the amplitudes of isolated IPSCs. Slices of $CBI^{+/+}$ mice pre-incubated in SR141716A (SR) showed no LTD. LFS 2 had no effect in $CBI^{-/-}$ mice. **c**, LTD was accompanied by increased PPF, which was absent in $CBI^{-/-}$ mice. Insets show representative traces before and after HFS or LFS (1, 2, respectively). Means \pm s.e.m. are shown.

mental adaptations to life-long absence of CB1, and cannot be easily attributed to the lack of CB1 during LTP induction. Low-frequency stimulation with 900 pulses at 1 Hz (LFS 1) of the same pathway induced a persistent decrease in excitatory synaptic transmission (long-term depression, LTD) in both *CB1*^{-/-} and *CB1*^{+/+} mice with no difference between genotypes (depression of population spike amplitude to $75 \pm 7\%$ in *CB1*^{-/-} compared with $80 \pm 7\%$ in *CB1*^{+/+} mice, $n = 9$, $P > 0.05$; Fig. 4a).

As several recent studies indicate an involvement of CB1 in GABA-mediated synaptic transmission in hippocampus^{16,17} and amygdala⁶, we next looked for possible differences in this process within the basolateral amygdala of *CB1*^{-/-} and *CB1*^{+/+} mice. Low-frequency stimulation with 100 pulses at 1 Hz (LFS 2) of the lateral amygdala close to the external capsule induced a significant suppression of isolated GABA_A receptor-mediated inhibitory postsynaptic currents (IPSCs) in principal neurons of the basolateral amygdala of *CB1*^{+/+} mice. This suppression lasted for more than 20 min (hereafter called long-term depression of IPSCs, LTD_i, to $66.7 \pm 5.4\%$, $n = 8$, $P < 0.05$; Fig. 4b). Importantly, LTD_i was blocked in *CB1*^{+/+} mice by SR141716A (5 μ M; Fig. 4b), showing an acute involvement of the endocannabinoid system in the development of LTD_i. The involvement of CB1 in LTD_i was confirmed in *CB1*^{-/-} mice in which LTD_i was completely abolished (to $110.1 \pm 13.8\%$, $n = 8$, $P < 0.01$ compared with *CB1*^{+/+}; Fig. 4b). Consistent with previous reports^{16,17}, suppression of GABA-mediated synaptic transmission also increased paired-pulse facilitation (PPF) in *CB1*^{+/+} ($P < 0.05$) but not in *CB1*^{-/-} mice (Fig. 4c), indicating a local CB1-dependent decrease in GABA release from axon terminals in *CB1*^{+/+} slices.

Extinction of aversive memories is thought to be an active mnemonic process². As a new memory, it shares several attributes with other steps of memory formation^{9,10,18}; however, there is increasing evidence that some cellular pathways are specifically involved in extinction, but not in acquisition or consolidation of fear memories^{15,19,20}. We demonstrated a specific involvement of CB1-mediated neurotransmission in extinction of aversive memories. In principle, the enhanced excitatory synaptic plasticity in *CB1*^{-/-} mice (LTP; Fig. 4a) might explain the prolonged maintenance of aversive memories observed in these animals (Fig. 1a, b, g). However, an enhanced LTP is expected to coincide with an increased initial freezing response in the first extinction trial²¹, which was not observed in *CB1*^{-/-} mice. Accordingly, acute blockade of CB1 by a selective antagonist failed to affect LTP induction as well as acquisition and consolidation of the aversive memory. In contrast, the same approach revealed a significant involvement of CB1 in extinction (Fig. 2a). Tone-induced recall of the aversive memory was accompanied by an activation of the endocannabinoid system within the BLAC (Fig. 3), which possibly leads to a decrease of GABA-mediated transmission in a CB1-dependent manner (LTD_i; Fig. 4b, c).

The role of GABA-mediated transmission for extinction is, however, controversial^{22,23}. Within the amygdala, CB1 immunoreactivity was detected in a distinct subset of GABA-containing interneurons of the BLAC⁶ (one of the sites where aversive memories might be formed and stored²⁴), but not in the central nucleus of the amygdala⁶ (the principal output site of the amygdala¹). Taking into consideration that principal neurons of the BLAC and neurons of the central nucleus of the amygdala might be inversely correlated in their activities^{25,26}, we propose that the CB1-mediated decrease of activity of local inhibitory networks within the BLAC leads to a disinhibition of principal neurons and finally to extinction of the freezing response. The selective and locally restricted inhibition of GABA-mediated transmission might not be easily reproduced by systemic administration of GABA-interfering drugs^{22,23}. Thus, future studies will have to confine such treatments to the BLAC to validate that CB1-mediated inhibition of GABA-mediated transmission is indeed crucially involved in the extinction of

aversive memories mediated by CB1. It remains to be shown whether CB1 is not only involved in extinction of aversive memories but also in adaptation to aversive situations in general and/or in extinction of memories, independently from their emotional value.

Overall, our findings suggest that the endogenous cannabinoid system could represent a therapeutic target for the treatment of diseases associated with inappropriate retention of aversive memories or inadequate responses to aversive situations, such as post-traumatic stress disorders², phobias, and certain forms of chronic pain¹¹. □

Methods

Animals

Adult male C57BL/6J OlaHsd mice (6–8 weeks; Harlan-Winkelmann) and male *CB1*^{-/-} and *CB1*^{+/+} littermates (10–16 weeks; see Supplementary Information) were housed individually with an inverse 12/12 h light/dark cycle (lights off at 8:00) for at least 2 weeks before starting the experiments.

Behavioural studies

Experimental procedures were approved by the Committee on Animal Health and Care of local Government. Experiments were performed between 9:00 and 14:00. Animal's behaviour was analysed in a blind fashion with regards to genotype and drug treatment. Data were analysed by analysis of variance (ANOVA) followed by Fisher's least significant difference test for planned comparisons, Mann-Whitney *U*-test or unpaired Student's *t*-test. A *P*-value of < 0.05 was considered statistically significant. Experimental procedures for pain threshold and unconditioned freezing, elevated plus maze and open field are described in Supplementary Information.

Fear conditioning

For conditioning, animals were placed into conditioning chambers (MED Associates). After 3 min, a 20-s tone (9 kHz, 80 dB) was presented that co-terminated with a 2-s electric foot-shock (0.7 mA). In pharmacological experiments animals received a 1-s shock to avoid ceiling effects in the freezing response due to the combination of foot-shock and injection stress. Animals were returned to their home cages 60 s after shock application. At the given time points after conditioning, animals were placed into transparent plexiglas cylinders that differed from the conditioning context, and a 60-s or 180-s tone was presented 3 min later (extinction trials). Animals were returned to their home cages after another 60 s. Mice were experimentally naive except for the stronger extinction protocol, where they had been tested on the elevated plus maze 5 days before. Freezing behaviour (defined as the absence of all movements except for respiration) was quantified from videotapes by trained observers that were blind to genotype and drug treatment, and data were normalized to the respective observation periods.

Pharmacological treatment

SR141716A (NIMH Chemical Synthesis and Drug Supply Program) was dissolved in vehicle solution (1 drop of Tween-80 in 3 ml 2.5% dimethylsulphoxide in saline). SR141716A (3 mg per kg body weight) and vehicle were injected subcutaneously at 20 ml per kg body weight under light isoflurane anaesthesia.

Measurement of endocannabinoids

C57BL/6J OlaHsd mice were randomly assigned to three groups ($n = 20$ each). On the conditioning day, two groups were conditioned as described before (paired). The remaining group received the foot-shock first and a 20 s tone 3 min later (unpaired). On the next day, all animals were placed into the cylinders, but only one of the paired groups and the unpaired group were exposed to a 3-min tone. Immediately after the end of the tone (or equivalent time in cylinder), animals were killed, brains were quickly removed and snap-frozen in isopentane/dry ice. mPFC and BLAC were punched from the frozen brain using a cryocut and cylindrical brain punches (Fine Science Tools, internal diameter 2.0 mm and 0.8 mm, respectively). Length of punches was approximately 1.6 mm for mPFC (start: bregma +2.8 mm²⁷) and 1.2 mm for BLAC (start: bregma -1.0 mm²⁷). Brain tissue of mPFC and bilateral BLAC, respectively, of 5 mice was pooled to obtain a single data point. Tissues (10–15 mg per data point) were dounce-homogenized with chloroform/methanol/Tris-HCl 50 mM, pH 7.4 (1/1/1 by volume) containing 5 pmol of octa-deuterated (*d*₈)-anandamide and 50 pmol of *d*₈-2-arachidonoylglycerol (Cayman Chemicals) as internal standards. Lipid-containing organic phase was dried down, weighed and pre-purified by open-bed chromatography on silica gel, and analysed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) using a Shimadzu high-performance liquid chromatography (HPLC) apparatus (LC-10ADVP) coupled to a Shimadzu quadrupole mass spectrometer (LCMS-2010) via a Shimadzu APCI interface. Mass spectrometry analyses were carried out in the selected ion-monitoring (SIM) mode as described previously²⁸. Temperature of the APCI source was 400 °C; HPLC column was a Phenomenex (5 μ m, 150 \times 4.5 mm) reverse phase column, eluted as described²⁸. Anandamide (retention time of 14.5 min) and 2-AG (retention time of 17.0 min) quasi-molecular ions were quantified by isotope dilution with the above-mentioned deuterated standards²⁸ and their amounts in pmols normalized per mg of lipid extract. Data were statistically evaluated by ANOVA.

Electrophysiology

Brain slices were prepared essentially as described²⁹. IPSCs and population spikes were evoked by square pulse stimuli (0.066 Hz, 5–12 mA, 200 µs) delivered by means of bipolar tungsten electrodes positioned within the lateral amygdala close to the external capsule. Population spikes were recorded in the basolateral amygdala close to lateral amygdala using glass microelectrodes (2–3 MΩ) filled with artificial cerebrospinal fluid (ACSF)²⁹. HFS (five trains at 100 Hz for 1 s, 10-s interstimulus interval) was applied to induce LTP, and LFS1 (900 pulses at 1 Hz) was applied to induce LTD. Whole-cell GABA-mediated currents were isolated by adding NBQX (0.005 mM) and D-(-)-2-amino-5-phosphopentanoic acid (AP5; 0.05 mM) to ACSF (bubbled with 95% O₂/5% CO₂; pH 7.3), and were recorded from visually identified somata of principal neurons of the basolateral amygdala²⁹ by glass electrodes (4.5–5 MΩ)¹⁶ containing (in mM): Mg-ATP 2, CsCH₃SO₃ 100, CsCl 60, EGTA 0.2, HEPES 10, MgCl₂ 1, QX314 5 and Na₃GTP 0.3 (pH 7.3). Patch clamp experiments were performed at 24 ± 1 °C at a holding potential of –70 mV. LTD was induced by 100 stimuli at 1 Hz (LFS 2). PPF was induced as described³⁰. Data are expressed as means ± s.e.m. We tested significance using the Student's *t*-test.

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The authors declare that they have no competing financial interests.

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A transcription factor response element for gene expression during circadian night

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Mammalian circadian clocks consist of complex integrated feedback loops^{1–10} that cannot be elucidated without comprehensive measurement of system dynamics and determination of network structures¹¹. To dissect such a complicated system, we took a systems-biological approach based on genomic, molecular and cell biological techniques. We profiled suprachiasmatic nuclei and liver genome-wide expression patterns under light/dark cycles and constant darkness. We determined transcription start sites of human orthologues for newly identified cycling genes and then performed bioinformatical searches for relationships between time-of-day specific expression and transcription factor response elements around transcription start sites. Here we demonstrate the role of the Rev-ErbA/ROR response element in gene expression during circadian night, which is in phase with *Bmal1* and in antiphase to *Per2* oscillations. This role was verified using an *in vitro* validation system, in which cultured fibroblasts transiently transfected with clock-controlled reporter vectors exhibited robust circadian bioluminescence¹².

To perform comprehensive measurement of mammalian circadian gene expression, we profiled genome-wide expression patterns of central (suprachiasmatic nuclei, SCN) and peripheral (liver) clocks every four hours during light/dark cycles (LD) or constant darkness (DD) over two days. We extracted total RNA from 50 pooled SCNs and four pooled livers at each time point, prepared biotinylated complementary RNA and used an Affymetrix mouse high-density oligonucleotide probe array (GeneChip) to determine SCN and liver gene expression.

The data obtained were analysed through two statistical cosine filters, one for LD and the other for DD time courses (see

Exhibit D

PENDING STUDY

*An FDA-Approved Investigation of the
Safety and Efficacy of Medical
Marijuana in Veterans with Chronic,
Treatment-Resistant Posttraumatic
Stress Disorder*

By: Sue Sisley
Principal Investigator

The Use of Medical Cannabis to Treat PTSD

by

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I. Introduction

Currently, there are approximately 500 suicides a month in patients with Post Traumatic Stress Disorder (PTSD) and all other causes, and over three hundred thousand backlogged disability claims involving PTSD and depression. n1. Those suffering from PTSD also have a reduced quality of life, an increased number of hospitalizations, high frequency of depressions and alcohol drug abuse, and suffer in social, family, and work life. n2. For patients who are treated, many have poor responses to psychotherapy and pharmacological treatment and often turn to alcohol and drugs. n3.

Recent studies demonstrate the potential benefits of the use of cannabis for PTSD. These studies confirm that extinction of aversive memories and the adaptation to stress responses are in part, controlled by endocannabinoids. n4. There are two cannabinoid receptors in the brain, CB1 and CB2. These receptors are activated by: endocannabinoids, which are synthesized internally in the body, cannabinoids derived from the cannabis plant (such as THC), and synthetic cannabinoids that are synthesized in a laboratory. This natural system works much like our natural GABA system. Just as we produce our own endocannabinoids, we produce our own internal GABA, and we use synthetic benzodiazapines that bind to the receptors. Likewise, we have cannabinoid receptors, and we should be using cannabis to modulate them. Cannabinoids can act as a therapeutic target for the treatment of diseases associated with the inappropriate retention of aversive memories, such as PTSD. n5 Furthermore, because of the effects of the cannabis on the stress response, it is likely that potential patients treated with cannabinoids may also benefit from the stress-reversing effects of the drug. n6

While the state of Arizona has acknowledged and approved the use of cannabis for many physical illnesses such as multiple sclerosis and chronic pain, it has failed to acknowledge the use of cannabis for psychological disorders such as PTSD, in which the medical benefits of cannabis are scientifically proven. This reflects unfounded discrimination on mental illness and psychological disorders. As Nancy Pelosi stated in a recent address on health care, "Illness of the brain must be treated just like illness anywhere else in the body." n7. Recently, the federal government has expressly acknowledged this in its passage of the Mental Health Parity and Equalization Act of 2008, mandating that health care providers provide equal treatment for mental disorders/substance abuse disorders as it does for any other physical illness. n8 The stereotype that psychological illnesses are any less debilitating or credible than physical illnesses is unacceptable and has no basis in science or reality. In both cases people are sick and need care; in both cases there are treatments that can relieve them of pain. When

people receive the necessary treatment, people have the potential to get better and be productive and independent citizens. n9.

Hundreds of recent studies indicate that cannabis is an effective treatment for PTSD. Considering the high suicide rate associated with PTSD (50-100 suicides a month for veterans alone) n10, and that accepted psychotherapeutic and pharmacological treatments are often ineffective, n11, it is imperative that PTSD patients have access to another option that is effective, natural, safe, and can be regulated by a doctor. These are people, often veterans, whose chronic psychological trauma, depression, insomnia, and accompanying symptoms cannot be relived by conventional therapy or psychotherapeutics and is worsened by alcohol. n12. In fact, since the U.S. sent more than 1.6 million men and women into combat in Iraq and Afghanistan in 2001, 18.3% of those returning have PTSD or major depression. n13. These patients have fought for our country and are now plagued with horrible memories. Their health and quality of life should be of top priority, and studies show and patients have testified that cannabis is an effective, alternative treatment. Cannabis can help relieve these patients of psychological trauma, it can stop horrible nightmares and stress related sleep disorders, and it can provide them with a better quality of life. n14

II. The Effectiveness of Cannabis as a treatment for PTSD.

a) The endocannabinoid system reverses enhancing effects of stress and helps with retention of aversive memories.

Over the past few years, remarkable advances have been made in our understanding of the endocannabinoid system and its molecular and physiological functions. n15. The potential therapeutic value of cannabinoid modulation is highlighted by the dense expression of the cannabinoid CB1 receptor in regions known to be significant for anxiety and emotional learning, particularly the basolateral amygdala (BLA). n16.

The endocannabinoid system has specific involvement in the habituation component of fear extinction and mediates habituation to repeated stress, suggesting that augmentation of endocannabinoid signaling is a good target for the treatment of affective disorders. n18, n19. The endocannabinoid system has a direct effect on the natural brain's function of dealing with information and can in fact aid the brain in discarding unneeded information. n20.

The functions of the endocannabinoid system are especially relevant to the treatment of conditions associated with retention of aversive memories and stress related disorders, such as PTSD. A recent study examining the cannabinoid receptor activation in the BLA found that it reverses the enhancing effects of environmental stress on inhibitory avoidance (IA) conditioning and its impairing effects on extinction. n21. The study tested rats, known for their love of dark places, who were given electric shocks when entering the darkened region of their cage. Shortly thereafter, the rats became afraid of the dark area and began to remain in the brighter part of the cage. The researchers then

stopped giving the electric shock treatment and the rats returned to the dark area. The length of time between the shocks stopping and the rats returning was measured. In the next phase of the study, a new group of rats were used. These rats were shocked as they entered the dark area of the cage and were placed on an elevated grid. (Most animals, including rats, avoid walking over elevated grids as they find the distressing). It took longer for this group of rats to trust the dark region again. The researchers then tested a third group of rats, who were treated in the same way as the second group, except in this group a synthetic THC-like compound was injected into the BLA, the region of their brains associated with fear. This medical-marijuana receiving group of rats returned just as quickly to the dark spot in the cage as the rats in group one. n22.

The beneficial effects of cannabinoids in the BA are extremely significant. Specifically, the study found that: 1) cannabinoid receptor activation in the BA blocks the effects of stress on the conditioning and extinction of inhibitory avoidance (IA); 2); cannabinoid receptor stimulation in the BLA reduces stress-induced elevations in corticosteroid levels (this is significant because most people with PTSD show a high secretion of cortisol), n23; and (3) the CB₁ receptor has an extremely important role in the BLA in the extinction of avoidance behavior because the receptor antagonist impairs IA extinction. n24. These findings show that cannabinoid receptor activation can act *to reverse the effects of stress on memory*. These results support a wide therapeutic application for the cannabis cannabinoids in the treatment of conditions in which patients suffer from aversive memories and stress. PTSD patients should be entitled to a treatment that can have such a profound beneficial effect on relieving traumatic memories.

b) Cannabinoids are effective in cessation of nightmares and a reduction in nightmare intensity

The disruption of sleep is often one of the most debilitating parts of PTSD and patients are often unable to find relief through pharmaceutical treatment. n25. Particularly, nightmares and sleep disorders are frequent symptoms of PTSD, with some patients experiencing even more severe problems such as violent or injurious behaviors during sleep, sleep paralysis, and hypnagogic and hypnopompic hallucinations. n26, n27.

Recent studies have shown that cannabis is effective in cessation of nightmares and reduction of nightmare intensity. In a study evaluating the effects of an endocannabinoid receptor agonist on treatment-resistant nightmares in patients diagnosed with PTSD, patients who had continued nightmares despite treatment with conventional anti-depressants and hypnotics were reviewed after treatment with nabilone, an endocannabinoid receptor agonist. n28. A large majority (72%) of patients experienced either cessation of nightmares or a significant reduction in nightmare intensity. n29. Furthermore, patients noted improvement in sleep time, the quality of sleep, and the reduction of daytime flashbacks and night sweats. n30.

These findings are extremely significant because they not only illustrate the many benefits of cannabis on PTSD symptoms, but also that cannabis can be an effective option for patients who are unable to find relief with the currently accepted treatments.

Dr. Tod Mikuriya, psychiatrist, author, and former marijuana research for the National Institute of Health, emphasized the importance of treating sleep deficits in those with PTSD when he explained, "PTSD often involves irritability and inability to concentrate, which is aggravated by sleep deficit. Cannabis use enhances the quality of sleep through modulation of emotional reactivity. It eases the triggered flashbacks and accompanying emotional reactions, including nightmares. The importance of restoring circadian rhythm of sleep cannot be overestimated in the management of PTSD." n31.

c) Cannabinoids promote neurogenesis and produce anxiolytic and antidepressant like effects.

The hippocampus is able to generate new neurons (neurogenesis) throughout the lifespan of mammals. n32. Studies teach us that newborn hippocampal neurons are functionally integrated into the existing circuitry and are positively correlated with learning and memory processes and the developmental mechanisms of stress and mood disorders. n33. Recent studies have shown that chronic treatment with synthetic cannabinoids produces antidepressant and anxiolytic effects. The anxiolytic effects are achieved by promoting hippocampal neurogenesis, which is in turn promoted by cannabinoids. n34. By finding that embryonic and adult rat hippocampal neural stem/progenitor cells are immunoreactive for CB1 cannabinoid receptors, studies demonstrate that cannabinoids can act on CB1 receptors to regulate neurogenesis. n35. This is further corroborated by findings that cannabinoids promote proliferation, but not differentiation, of embryonic hippocampal neural stem/progenitor cells via activation of CB1 receptors combined with G proteins and ERK signaling. n36.

The anti-depressant and anxiolytic effects of cannabis are important as anxiety and depression are frequent symptoms of PTSD and can be very debilitating. n37. It is well-founded that cannabis and its major psychoactive component, (-)-*trans*- Δ^9 -tetrahydrocannabinol, have profound effects on mood and can modulate anxiety and mood states. n38. Thus, stimulating the endogenous cannabinoid system with natural cannabinoids could be a major therapeutic target for the treatment of anxiety-related and mood disorders. n39. In a study that looked at treating anxiety with cannabinoids, blocking the CB1 receptor resulted in the rats having more fear, demonstrating that modulation may be useful treatment for blocking fear, as seen in the blockade mice. n40. These results indicate that the endocannabinoid system can be modulated to enhance emotional learning and that endocannabinoid modulators may be therapeutically useful for exposure based psychotherapies such as those used to treat PTSD and other anxiety disorders. n41.

Based on its efficacy alone, cannabis should be considered an acceptable treatment for PTSD. As Dr. Mikuriya said "Cannabis relieves pain, enables sleep, normalizes gastrointestinal function and restores peristalsis. Fortified by improved digestion and adequate rest, the patient can resist being overwhelmed by triggering stimuli. There is no other psychotherapeutic drug with these synergistic and complementary effects." n42. Dr. Mikuriya also emphasizes that cannabis can relieve many other symptoms of PTSD such as physical pain, fatigue, and sleep deficit.

Furthermore, restorative exercise and diet are requisite components of PTSD treatment and depression treatment, and cannabis, unlike some analgesics, sedatives, and benzodiazepines, does not leave the patient too immobile to exercise. n43.

III. PTSD and substance abuse

Many PTSD patients have poor responses to psychotherapy and often turn to alcohol and drugs. n44. Moreover, many suffer from chronic pain and become addicted to opiate pain medications. n45. Due to continuous problems such as depression, anxiety, secondary alcoholism, and substance abuse that PTSD patients suffer from and the numerous poor responses to pharmacological and psychological treatments, alternative treatments such as cannabis are imperative.

While many studies, and many State Departments of Health, cite cannabis use as substance abuse in PTSD patients, they ignore the positive effects of cannabis on the brain and the reality that patients may not be abusing cannabis, but using it as an alternative, effective treatment. Abuse can occur with any drug, including medically prescribed Oxycontin or Vicodin as well as an over the counter drug like Tylenol. But the possibility that these drugs can be abused does not make them illegal. The possibility that some people might abuse cannabis should not make it illegal, when, like these other drugs, it is scientifically proven to effectively treat a condition. In fact, "it is generally appreciated that the 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." n46.

The differing effects of cannabis and other drugs of abuse on the brain highlight the difference between using a drug as an effective treatment versus substance abuse. Chronic administration of the major drugs of abuse including opiates, alcohol, nicotine, and cocaine has been reported to suppress hippocampal neurogenesis in rats. n47. Unlike these major drugs that inhibit neurogenesis, studies demonstrate that cannabis *promotes* hippocampal neurogenesis. n48. This suggests a role of hippocampal neurogenesis in the initiation, maintenance, and treatment of drug addiction.

The specific effect of the cannabinoid system on the fear response is significant and suggests the potential for long-term relief. n49. Current acceptable treatments such as behavior therapy, on the other hand, are ineffective for many. While behavior therapy for human anxiety disorders is often effective, extinction-like treatments require repeated cue exposures and are vulnerable to reversal by a number of environmental factors, particularly stress. n50. Thus, cannabis has the potential to be an effective alternative to often-ineffective behavior therapy and extinction treatment. n51.

The ineffectiveness of currently acceptable treatments leads to substance abuse. Patients unable to find relief seek it elsewhere, with substances that are not regulated or monitored by a physician. Moreover, psychiatrist-advised use of medical marijuana can actually *help* PTSD patients reduce their alcohol intake. Marijuana addiction potential is

a fraction of that of alcohol (3% vs. 10%). n52. Dr. Christopher Fichtner, section chief for PTSD at Hines V.A. Hospital in Illinois, explained that the use of medical cannabis can reduce the physical and psychological harm for those who self-medicate with alcohol. n53.

IV. New Mexico: Taking the lead in treating PTSD with cannabis.

New Mexico has taken the lead in explicitly allowing people with PTSD to have access to marijuana under its medical marijuana law. PTSD accounts for more patients than any other of the state's 16 eligible debilitating conditions approved for medical marijuana treatment. n54. After a review of the evidence of the effectiveness of marijuana in treating PTSD, health professionals in New Mexico agreed that medical marijuana could be beneficial for patients with PTSD. On the other hand, health officials in Colorado are denying veterans and other patients suffering from PTSD a legitimate, safe, treatment alternative.

The chief medical officer of the Colorado health department said, "There is no evidence of efficacy of marijuana for treatment of PTSD in the medical literature." n50. This statement is outright false, inconsistent with evidence-based medicine and demonstrates ignorance of the hundreds of medical studies on the efficacy of marijuana for PTSD treatment. To deny the enormous body of medical literature is outrageous and offensive to the suffering PTSD patients who are now the victims of the health department's ignorance. Dr. Eve Elting, a New Mexico physician, emphasized the offensiveness of the Colorado Health Department when she said, "It's bad enough they have something that makes life so challenging. On top of that they're discriminated against, made to feel like they're doing something wrong." n55.

New Mexico is not alone in recognizing cannabis as an effective treatment for PTSD. In Canada, the government *pays* for medical marijuana for their veterans, acknowledging that for many, it is more effective than available alternatives, with fewer side effects. n56. In Israel, the Ministry of Health is currently granting licenses for people who have PTSD to use medical marijuana. n57

Even Croatia acknowledges cannabis as a treatment for PTSD. In 2009, Croatia's Supreme Court threw out a jail sentence given to a veteran who used marijuana for his PTSD. n58. This ruling is extremely significant considering Croatia's "zero tolerance" drug policy. In its ruling, the court noted that "the defendant suffers from PTSD, and marijuana relaxes him and helps him to overcome psychological problems." n59.

V. Conclusion

To deny those with PTSD suffering from psychological trauma and terrifying flashbacks access to a natural herb that is scientifically proven to provide them with relief is simply outrageous. By allowing PTSD to be treated with medical marijuana, physicians

can help patients treat their condition with cannabis and assist the patient in using cannabis in a manner that is safe and most effective for the particular patient. Physicians can be re-assured that there is an ample body of medical literature that supports the beneficial use of cannabinoids. Studies teach us that we have our own cannabinoid receptors in our internal cannabinoids, and these should be modulated as they are proven to reverse effects of stress and help with retention of aversive memories, promote neurogenesis, and can reduce nightmares, fear, anxiety, mood disorders and other PTSD symptoms. The importance of the endocannabinoid system and the large body of medical literature supporting the beneficial use of cannabis should be acknowledged. Without the acceptance of cannabis to treat PTSD, patients who cannot find relief with pharmaceuticals and psychotherapy are forced to turn to the streets to have access to cannabis. They are denied the very important role of the doctor in helping them treat their condition. These patients will often turn to substance abuse and many turn to suicide.

We are sending millions of our citizens to Iraq and Afghanistan, and many are coming back afflicted with PTSD and other psychological trauma. n60. We should give them all of the tools available to regain their health. The enormous volume of scientific research and data proves that the use of medical marijuana for PTSD is safe and effective. To deny patients access to a treatment whose efficacy is well founded with scientific evidence is callous and discriminatory at best.

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An FDA-Approved Investigation
of the Safety and Efficacy of
Medical Marijuana
in Veterans with
Chronic, Treatment-Resistant
Posttraumatic Stress Disorder

- By Sue Sisley, M.D.
- Principal Investigator

No personal, professional nor
financial interest in Marijuana

- I have NO personal experience
with MJ
- I do NOT write Qualifying
Patient certifications for MJ
- I do NOT receive money from
MJ Dispensaries or other MJ
Industries.

Study Objectives

- To investigate the safety and efficacy of five different potencies of marijuana
- (0%, 2%, 6%, or 12% THC or 6% THC/6% CBD),
as treatment in veterans diagnosed with chronic, treatment-resistant, service-related PTSD
- And to compare the safety and efficacy of two substance delivery methods, smoking or vaporizing.

U.S. Drug Enforcement Administration



- Schedule I
 - No medical benefit and high potential for abuse
- Schedule II
 - Medical benefit, strong abuse potential
- Schedule III
 - Accepted medical use, less abuse potential than above

Table 1.1 Examples of Drugs in the Five Scheduling Categories

Schedule I	Ecstasy, China White, GHB, Heroin (synthetic and natural), Lysergic Acid Diethylamide (LSD), Marijuana, Mescaline, Peyote, Psilocin and Psilocybin (constituents of magic mushrooms)
Schedule II	Amphetamine, Cocaine and Crack, Codeine, Fentanyl, Hydrocodone, Meperidine (Demerol [®]), Methadone, Methylphenidate (Ritalin), Morphine, Opium, Oxycodone (OxyContin [®] , Percocet [®]), Phencyclidine (PCP)
Schedule III	Anabolic steroids, Barbiturates, Ketamine, LSD precursors
Schedule IV	Alprazolam (Xanax [®]), Clonazepam (Klonopin [®] , Clonopin [®]), Diazepam (Valium [®]), Flunitrazepam (Rohypnol), Lorazepam (Ativan [®]), Triazolam (Halcion [®]), Zolpidem (Ambien [®])
Schedule V	Codeine preparations—200 mg/ml or 100 g (Cosanyl, Robitussin A-C [®] , Cheracol [®] , Cerase [®] , Pediacof [®])

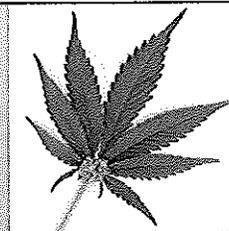
NIDA Monopoly

- The National Institute of Drug Abuse holds a government-enforced monopoly on the legal supply of research marijuana.

- Marijuana is the only Schedule 1 drug for which the federal government not only controls the supply, but also requires a special review of all scientific protocols by a NIDA/Public Health Service (PHS) review panel.



NIDA Monopoly



- Since 1968 the DEA has licensed only one

Cannabis-production facility in the US, housed at the University of Mississippi

- If NIDA decides not to sell Marijuana to a group of researchers, their study becomes impossible to conduct
- Unlike the 30-day timetable that the FDA must follow when reviewing research protocols, the NIDA/PHS review process has NO DEADLINE.

NIDA Monopoly



- We unsuccessfully tried to purchase 10 grams of marijuana from NIDA for 7 years for vaporizer research.
- Our LSD study looking at Tx of End-of-Life Anxiety had NO delay once FDA-approval obtained. No 2nd review required by DEA/NIDA —only Cannabis has this situation.

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A15

Marijuana May Be Studied for Combat Disorder

By DAN FROSCHE

DENVER — For years now, some veterans groups and marijuana advocates have argued that the therapeutic benefits of the drug can help soothe the psychological wounds of battle. But with only anecdotal evidence as support, their claims have yet to gain widespread acceptance in medical circles.

Now, however, researchers are seeking federal approval for what is believed to be the first study to examine the effects of marijuana on veterans with chronic, post-traumatic stress disorder.

The proposal, from the Multidisciplinary Association for Psychedelic Studies in Santa Cruz, Calif., and a researcher at the University of Arizona College of Medicine, would look at the po-



“There is a widely accepted need for a new treatment of PTSD,” said Rick Doblin, who wants to do research on marijuana.

condition, according to the state’s health department. It is unclear how many are veterans.

One recent Army veteran from Texas who fought in Iraq for 18 months beginning in 2006, said he used marijuana three times a day in lieu of the painkillers and anti-depressants he was prescribed after returning home. He asked that his name not be used because Texas does not allow medical marijuana.

The veteran, who said he had been shot in the leg and suffered numerous head injuries from explosions while deployed as a Humvee gunner, said marijuana helped quiet his physical and psychological pain, while not causing the weight loss and sleep deprivation brought on by his prescription medications.

Why Study New Treatments for PTSD?

- PTSD plagues between 6 and 10% of the US population at some point during their lifetime
- Approximately 18% of soldiers returning from combat in the Iraq war will have PTSD
- In 2010, the U.S. Veterans Administration spent about \$5.5 billion on PTSD disability payments to about 275,000 veterans

GLOBAL EDITION
The New York Times

Drugs Found Ineffective for Veterans' Stress

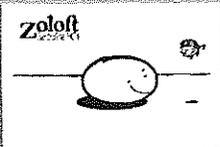


U.S. Marine waited to take psychological tests at the Marine Corps Air Ground Combat Center in Twentynine Palms, Calif., in 2009.
By BENJAMIN CAHLEY
Published Aug. 12, 2011

"Drugs widely prescribed to treat severe PTSD symptoms for veterans are no more effective than placebos and come with serious side effects."

Why Study New Treatments for PTSD?

A significant percentage of PTSD patients fail to respond adequately to FDA-approved treatments, suggesting a need to develop innovative treatments

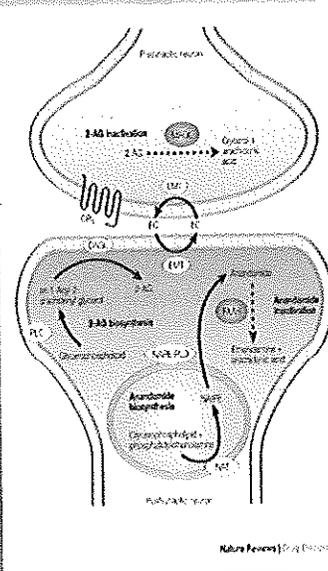


Why Study New Treatments for PTSD?

- Numerous anecdotal reports of marijuana used successfully for PTSD by veterans
- One recent Army veteran from Texas who fought in Iraq for 18 months beginning in 2006, said he used marijuana three times a day in lieu of the painkillers and antidepressants he was prescribed after returning home. (The New York Times, July 18, 2011)

Why Study New Treatments for PTSD?

- **The Endocannabinoid System** has been suggested to be involved in regulating sleep, anxiety, attention to and response to stressful situations; may be involved in the extinction of conditioned fear; modulate GABAergic transmission and enhance the release of endogenous opioids.
- Exogenous cannabinoids trigger the **Endocannabinoid System**
- Benzodiazepines and related GABA agonists used to treat anxiety and sleep disruption are not always tolerated and can produce physical dependence



Why Marijuana for PTSD?

- At present, there are NO PUBLISHED DATA from a randomized, placebo-controlled, study of the risks and benefits of marijuana for subjects with chronic treatment-resistant PTSD from any cause

- Our study is the only study of which we are aware attempting to conduct such a study

Timeline

- November 12, 2010

 - Protocol submitted to the FDA



- Morning April 28, 2011

 - Protocol approved by FDA

- Afternoon April 28, 2011

 - Protocol sent to DEA

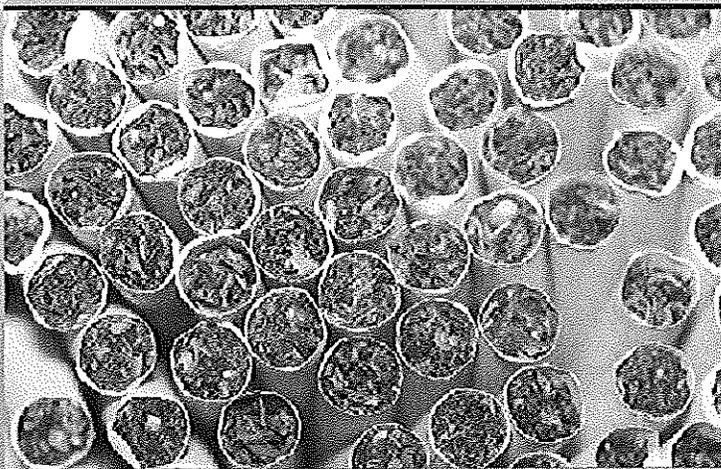


- 19 months later and still no reply from DEA/NIDA

Huge barrels store the end product, a ground-up mix of buds, leaves and stems that will eventually be machine rolled into joints, packaged in metal containers, and sent to the four remaining medical-marijuana patients still supplied by the federal government.



NIDA Marijuana Cigarettes



Unrolled NIDA Marijuana:
Mostly Leaf



Stems and Seeds in 3 NIDA-Supplied
Marijuana Cigarettes



Hypotheses

- Marijuana will ease the symptoms of PTSD, specifically reducing nightmares, improving sleep, and improving mood as measured in by the Clinician Administered PTSD Scale (CAPS)
- Marijuana, in a dose dependent manner, will ease the symptoms of PTSD
- Marijuana with 6% THC and 6% CBD will be more effective than marijuana with 6% THC alone

Participants

● 50 veterans with chronic treatment-resistant PTSD arising from their service in the US armed forces and with duration of PTSD symptoms lasting at least six months

● Recruitment:

- Letters of referral
- Contact with veterans' organizations
- Advertisements or announcements
- Word of mouth



Inclusion Criteria

- Meet **DSM-IV** criteria for chronic PTSD \geq 6 mo.
- **CAPS score \geq 50**
- Have had unsuccessful treatment with:
 - SSRI, SNRI, or MAOI
 - Any form of psychotherapy
- Agree not to change current psychotherapy or pharmacotherapy during study duration
- Agree not to participate in any other clinical trials during the study

Exclusion Criteria

- Pregnant or nursing
- History of or current **primary psychotic disorder or bipolar affective disorder type 1**
- Diagnosed with **dissociative identity disorder** or an **eating disorder** with active purging
- Diagnosed with **borderline personality disorder**
- Have evidence of significant uncontrolled hematological, endocrine, cerebrovascular, cardiovascular, coronary, pulmonary, gastrointestinal, or neurological disease

Exclusion Criteria

- Have **allergies** to marijuana
- Would present a **serious suicide risk** as assessed by the investigators using the Columbia Suicide Severity Rating Scale (C-SSRS)
- Meet DSM-IV criteria for **substance abuse** or dependence for any substance other than caffeine or nicotine in the past 60 days
- Are not able to give adequate informed consent
- **Used marijuana within a month of starting the study**
- Fail the initial urine **drug screen** and blood test which tests for illicit drug use within the prior month

Outcome Measures

- Clinician-Administered PTSD Scale (CAPS)
- Posttraumatic Diagnostic Scale (PDS)
- Beck Depression Inventory (BDI-II)
- Global Assessment of Function (GAF)
- Pittsburgh Sleep Quality Index (PSQI)
- Experiences with Self-Administering
- **○ Marijuana Survey (ESAMS)** – a sponsor-developed self-report instrument

Safety Measures

- Two four-hour long sessions on two consecutive days as an added safety measure prior to initiation of treatment phase
- Columbia Suicide Severity Rating Scale
- Brief Symptom Inventory
- Collection of vital signs during visit to study site
- Collection of any Adverse Events and Serious Adverse Events
- Participants are only given a 1 week supply (2 grams per day) at a time

Marijuana Doses

Stage 1

Dose	Number of Participants receiving dose	Smoked marijuana	Vaporized Marijuana
0% THC marijuana	10	5	5
2% THC marijuana	10	5	5
6% THC marijuana	10	5	5
12% THC marijuana	10	5	5
6% THC/6% CBD marijuana	10	5	5

Stage 2

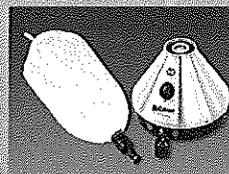
Dose	Number of Participants receiving dose	Smoked* Marijuana	Vaporized* Marijuana
6% THC marijuana	20	10	10
12% THC marijuana	10	5	5
6% THC/6% CBD marijuana	20	10	10

Marijuana Administration

● Method 1: Participant smokes pre-rolled marijuana cigarette, weighing 0.9 grams



● Method 2: Participant vaporizes material from pre-rolled cigarette in a Volcano vaporizer

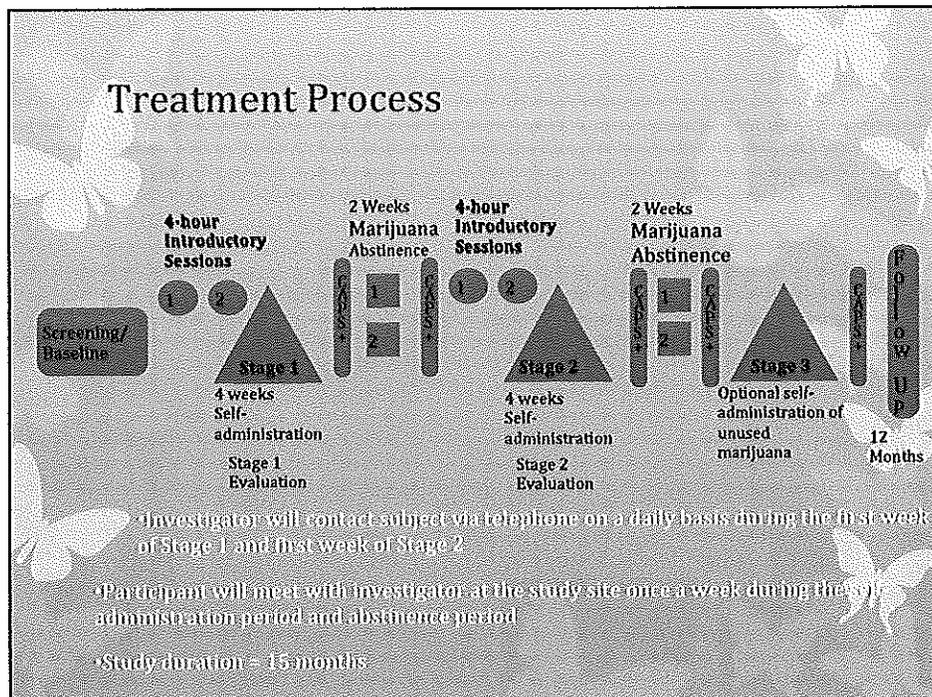


● Vaporize: to convert into vapor, by the application of hot air with no combustion

Benefits of Vaporizing

• No products of combustion, smoke, or taste of smoke

• Vapor still contains particulate matter from the plant, but compared to smoking it is much safer



Ensuring safety

- Storage box with combination lock
- Subjects required to return unused amounts to investigators during each weekly visit
- Should subjects request it, all unused marijuana will be returned to them at the end of Stage 2, reducing likelihood of unnecessary use or diversion
- Investigators provide subjects with portable video cameras, and subjects are asked to record their use of marijuana.
- Participant designates **an secondary verifier** who the investigator will telephone weekly
- Research staff will **contact subject via telephone daily for the first week of marijuana self-administration**
- Subjects provided with 1 week supply at a time (2 grams per day)

NIDA Monopoly

- In 2001, MAPS began helping Prof. Lyle Craker, director of the Medicinal Plant Program at the University of Massachusetts at Amherst, to apply to the DEA for a license to open a marijuana-production facility for FDA-approved research
- Craker's proposed facility would focus on providing medical-grade marijuana, including strains with CBD, to FDA-approved studies.

NIDA Monopoly

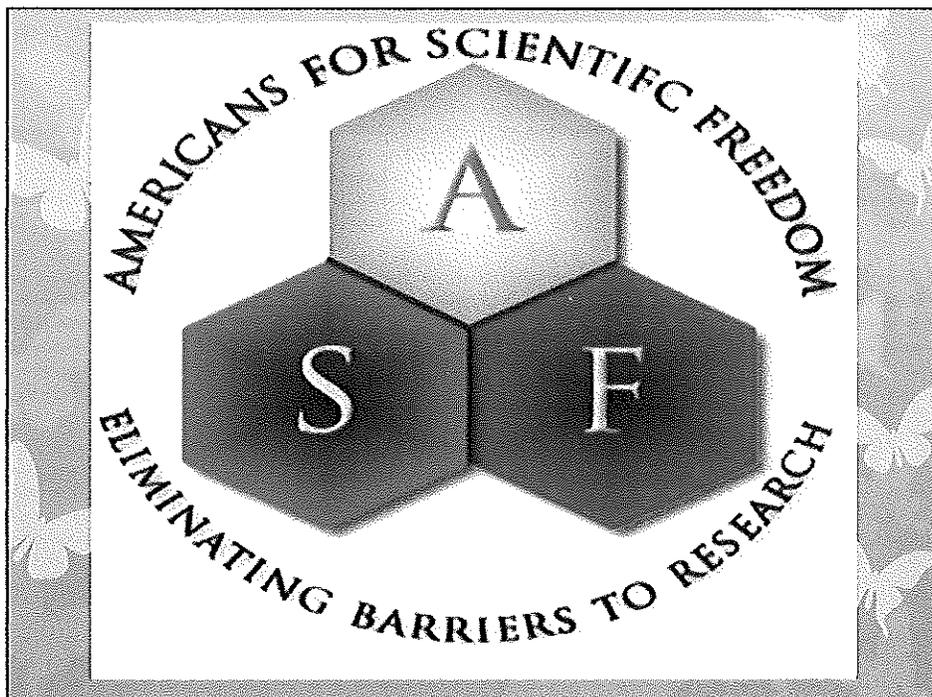
- In February 2007, the DEA's Administrative Law Judge, Mary Ellen Bittner, issued a recommendation affirming that it would be in the public interest for DEA to grant Prof Craker a license.
- According to Bittner's ruling, federal officials had repeatedly refused to provide marijuana on reasonable cannabis-research requests in a timely manner or had denied them outright, creating a clear need to end the NIDA monopoly

NIDA Monopoly

- Judge Bittner's recommendation was ignored for nearly two years.
- Six days before President Obama was inaugurated, Acting DEA Administrator Michelle Leonhard officially rejected the recommendation and denied Craker's license

NIDA Monopoly

- On December 15, MAPS filed with the First Circuit Court of Appeals
- On March 22, 2012, after three requests for delays, DEA responded.
- Professor Craker's lawyers responded in Spring and awaiting response from Courts.
- All the delay is victory for DEA, even if they lose in the end



Americans for Scientific Freedom (ASF)

- The purpose of the Americans for Scientific Freedom (ASF) is to coordinate rigorous, scientific studies to assess the safety and efficacy of cannabis and cannabis compounds for treating medical conditions.

The Institute will coordinate and support cannabis research throughout the State of Arizona and across the US. Research will focus on the potential medicinal benefits of cannabis for diseases and conditions as specified by the National Academy of Sciences, Institute of Medicine Report (1999) and by the Workshop on the Medical Utility of Marijuana, National Institutes of Health (1997).

The ASF will strive to conduct high-quality, controlled scientific studies intended to ascertain the general medical safety and efficacy of cannabis and cannabis products and examine alternative forms of cannabis administration. The Institute will be seen as a model resource for health policy planning by virtue of its close collaboration with federal, state, and academic entities. The ASF will also highlight the current barriers to federally-regulated Marijuana Research.

Help ASF become a SuperPAC

<http://americansforscientificfreedom.com/>

- a PAC submitting over 500 individual PAC contributions of \$10 or more collected within a one-year timeframe in order to earn the designation.
- SuperPAC status permits ASF to contribute significantly more to candidates.

ArMA and MCMS Resolutions to Eliminate Barriers to MJ Research

- THE ARIZONA MEDICAL ASSOCIATION, INC.
-
- HOUSE OF DELEGATES
- DATE: 5/15/12
-
- INTRODUCED BY: MARICOPA COUNTY MEDICAL SOCIETY
-
- SUBJECT: NIDA'S SOLE AUTHORITY AND BARRIERS TO THE ADVANCEMENT OF ESSENTIAL CANNABIS RESEARCH
-
- ASSIGNED TO: REFERENCE COMMITTEE ON REPORTS AND RESOLUTIONS

Exhibit E

STUDY

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

By: Drs. Eti Ganon-Elazar and Irit Akirav
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September 9, 2009

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 CB₁ 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 CB₁/CB₂ receptor WIN55,212-2 [*R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrol[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 CB₁ receptor in the BLA is crucially involved in the extinction of IA, because the CB₁ 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).

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 CB₁ 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 CB₁ 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

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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 $22 \pm 2^\circ\text{C}$ 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 CB₁/CB₂ receptor agonist WIN55,212-2 [*R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrol[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 CB₁ 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 $\mu\text{g}/0.5 \mu\text{l}$, 5 $\mu\text{g}/0.5 \mu\text{l}$, or 10 $\mu\text{g}/0.5 \mu\text{l}$. AM404 was used at 200 $\text{ng}/0.5 \mu\text{l}$ or 800 $\text{ng}/0.5 \mu\text{l}$, and AM251 was used at 6 $\text{ng}/0.5 \mu\text{l}$. 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 MgSO₄ 10% ethanol, 39.1% v/v propylene glycol, 0.98% w/v sodium pentobarbital, and 4.2% w/v chloral hydrate), restrained in a stereotaxic 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 CB₁ 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 \times 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 \times 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 \times 25 \times 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 \pm 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 CB₁/CB₂ 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 μ g/0.5 μ l) were pooled for all analyses (vehicle, $n = 12$). For WIN55,212-2 (5 μ g/0.5 μ l) 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 \times days (4 \times 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. 2a). 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).

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 μ g/0.5 μ l (WIN_2.5), $n = 7$] or a higher [10 μ g/0.5 μ l (WIN_10), $n = 7$] dose of WIN55,212-2 into the BLA after Ext1. Repeated-measures ANOVA [treatment \times days (3 \times 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. 2b). 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 2a, 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 CB₁/CB₂ 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

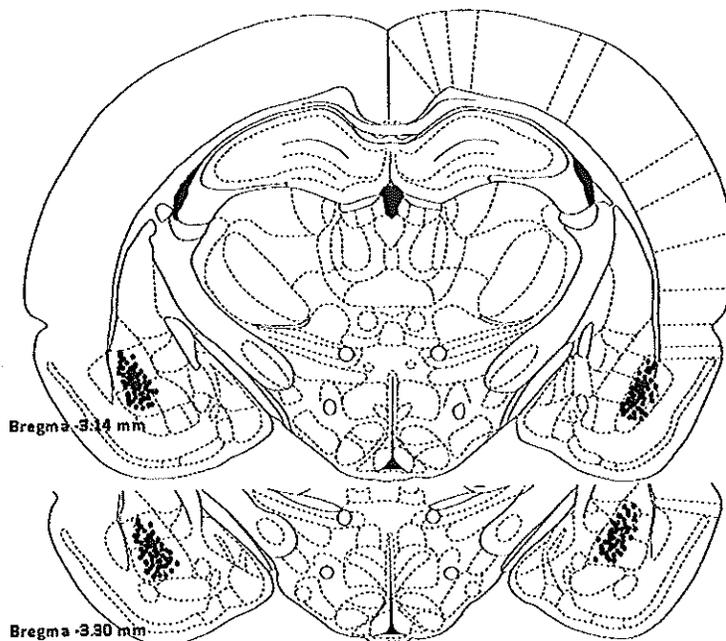


Figure 1. Representative schematic drawing of cannulae tip positions in the BLA. A coronal view at position 3.14 and 3.30 mm posterior to bregma.

[treatment \times days (4 \times 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. 2c). 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 μ l) 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 CB₁ receptor in the BLA is essential for IA conditioning or extinction. Hence, rats were microinjected with vehicle or the CB₁ 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 \times days (4 \times 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. 2d). *Post hoc* comparison unveiled a significant difference between the vehicle group and the groups microinjected with AM251 before conditioning (Pre-

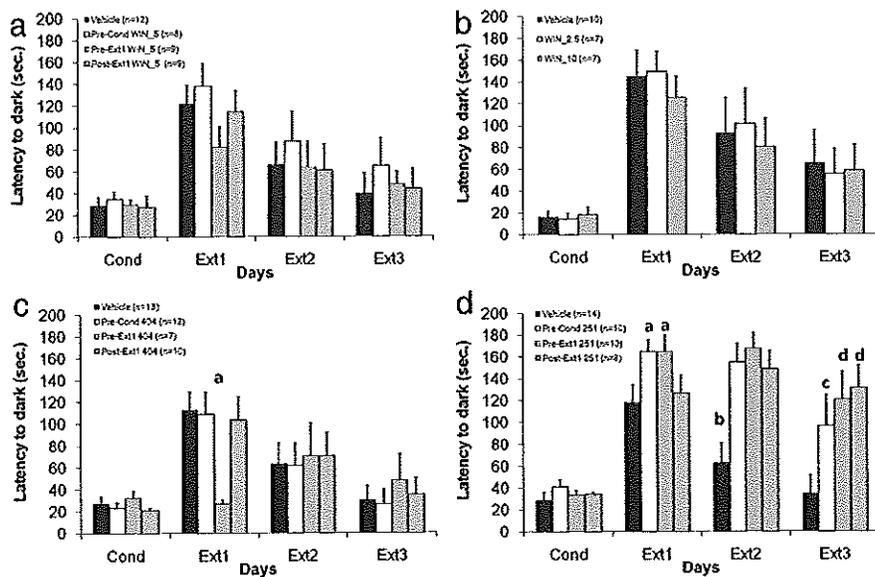


Figure 2. Cannabinoid receptor agonist WIN55,212-2 microinjected into the BLA has no effect on inhibitory avoidance conditioning or extinction. *a*, Rats were microinjected into the BLA with vehicle ($n = 12$), with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) before conditioning (Pre-Cond WIN_5, $n = 8$), 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 ($2.5 \mu\text{g}/0.5 \mu\text{l}$; WIN_2.5, $n = 7$) or a higher ($10 \mu\text{g}/0.5 \mu\text{l}$; 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 ($200 \text{ ng}/0.5 \mu\text{l}$) 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, $^*p < 0.01$) (for details, see Results). *d*, Rats were microinjected into the BLA with vehicle ($n = 14$) or AM251 ($6 \text{ ng}/0.5 \mu\text{l}$) 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, $^*p < 0.05$, 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 ($5 \mu\text{g}/0.5 \mu\text{l}$) and immediately afterward placed on the EP for 30 min (WIN_5+EP, $n = 7$). Repeated-measures ANOVA [treatment \times 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 ($5 \mu\text{g}/0.5 \mu\text{l}$) and immediately afterward placed on the EP for 30 min (WIN_5+EP, $n = 10$). Repeated-measures ANOVA [treatment \times 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 Post-Ext1, $n = 8$), or microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) and immediately afterward placed on the EP for 30 min (WIN_5+EP, $n = 8$). Repeated-measures ANOVA [treatment \times 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. 3c). 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 ($2.5 \mu\text{g}/0.5 \mu\text{l}$) 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 \times 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. 3d). Also, there were no within-subject differences in latency between the days ($F_{(1,21)} = 2.7$; NS), nor was there an

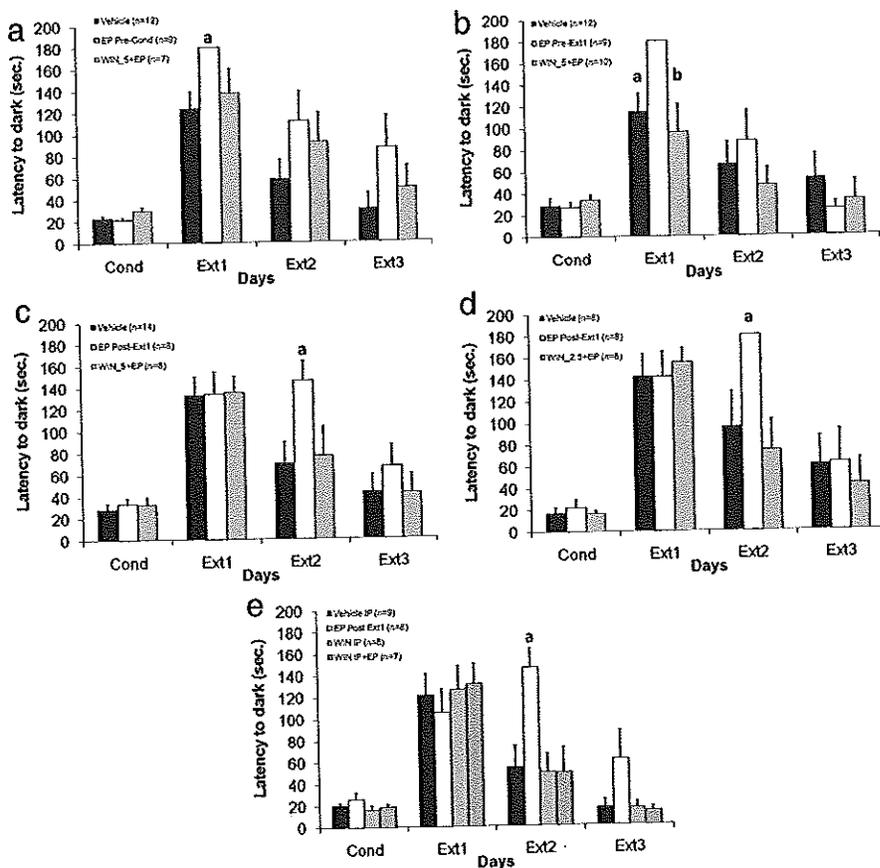


Figure 3. Cannabinoid receptor agonist WIN55,212-2 blocks the effects of EP stress on IA conditioning and extinction. *a*, Before conditioning, rats were microinjected with vehicle ($n = 12$), placed on the EP (EP Pre-Cond, $n = 9$), or microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) 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, $^*p < 0.01$). 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 ($5 \mu\text{g}/0.5 \mu\text{l}$) 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, $^*p < 0.05$, EP differs from vehicle; $^*p < 0.01$, 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 ($5 \mu\text{g}/0.5 \mu\text{l}$) 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, $^*p < 0.05$). 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 ($2.5 \mu\text{g}/0.5 \mu\text{l}$) 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, $^*p < 0.01$, 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, $^*p < 0.01$). Thus, intraperitoneal administration of WIN55,212-2 before stressor exposure also reversed the disrupting effect of the stressor on IA extinction.

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,

Table 1. The effects of cannabinoid receptor agonists and antagonist microinjected into the BLA on locomotion and anxiety in the open-field test

	Vehicle (<i>n</i> = 6)	AM404 (<i>n</i> = 6)	WIN55,212-2 (<i>n</i> = 6)	AM251 (<i>n</i> = 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 CB₁ 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. 3e). 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 CB₁ 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

Table 2. The effects of cannabinoid receptor agonists and antagonist microinjected into the BLA on pain sensitivity

	Vehicle (<i>n</i> = 6)	AM404 (<i>n</i> = 6)	WIN55,212-2 (<i>n</i> = 6)	AM251 (<i>n</i> = 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 CB₁ 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 (<i>n</i> = 6)	EP (<i>n</i> = 5)	WIN55,212-2 + EP (<i>n</i> = 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 (<i>n</i> = 6)	EP (<i>n</i> = 5)	EP + WIN55,212-2 (<i>n</i> = 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.

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).

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

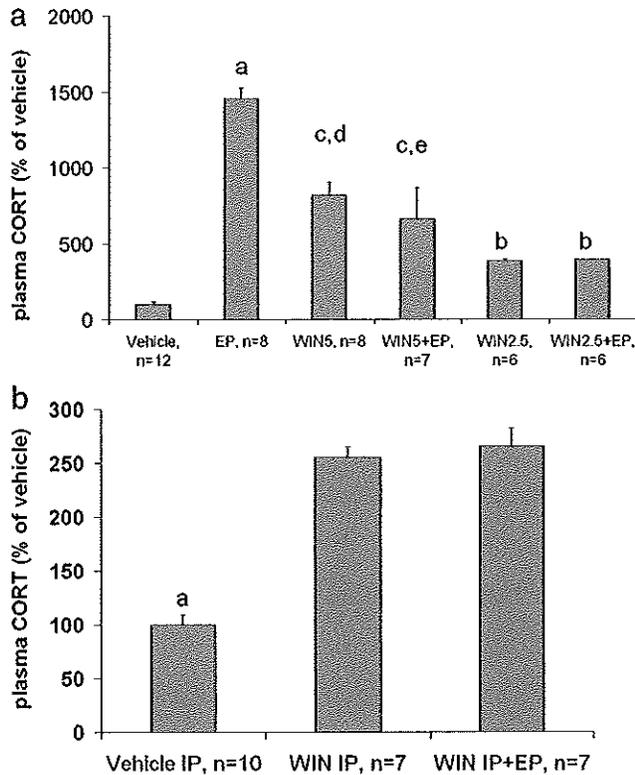


Figure 4. The effects of the cannabinoid receptor agonist WIN55,212-2 and EP stress on CORT levels. *a*, CORT levels were measured in rats microinjected with vehicle into the BLA (vehicle, $n = 12$), placed on the EP ($n = 8$), microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) into the BLA (WIN_5, $n = 8$), microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) into the BLA and placed on the EP (WIN_5+EP, $n = 7$), microinjected with a lower dose of WIN55,212-2 ($2.5 \mu\text{g}/0.5 \mu\text{l}$) 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 \pm SEM expressed as a percentage of the CORT values of the vehicle animals (CORT levels in the vehicle group, $95.52 \pm 16.7 \text{ ng/ml}$) ($p < 0.001$, 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 \pm SEM expressed as a percentage of the CORT values of the vehicle animals (CORT levels in the vehicle group, $381.01 \pm 64.39 \text{ ng/ml}$) ($p < 0.001$, 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 ($5 \mu\text{g}/0.5 \mu\text{l}$) into the BLA (WIN_5, $n = 8$), microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) and placed on the EP (WIN_5+EP, $n = 7$), microinjected with a lower dose of WIN55,212-2 ($2.5 \mu\text{g}/0.5 \mu\text{l}$) 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 ($2.5 \mu\text{g}/0.5 \mu\text{l}$ or $5 \mu\text{g}/0.5 \mu\text{l}$) 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, $381.01 \pm 64.39 \text{ ng/ml}$). 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), $199.8 \pm 40.8 \text{ ng/ml}$] compared with the vehicle group (Vehicle BLA, $n = 10$; CORT levels, $100 \pm 25.72 \text{ ng/ml}$) ($t_{(15)} = 2.16$; $p = 0.047$).

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 CB₁ receptor in the BLA in the extinction of avoidance behavior because the CB₁ 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 CB₁ receptor antagonist AM251 on inhibitory avoidance learning

Administration of the CB₁ 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 CB₁ receptors in the extinction of aversive memories has been substantiated by several groups in different behavioral paradigms using systemic administration. CB₁ 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 administering AM251 enhances acquisition of freezing behavior and impairs extinction in trace and delay pavlovian fear conditioning. However, several studies did not find the CB₁ 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 CB₁ 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 $\mu\text{g}/0.5 \mu\text{l}$, 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, Kobilko 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 CB₁ 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, CB₁ 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 CB₁ receptor activation in the amygdala, because the combination of restraint stress and CB₁ 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 $\mu\text{g}/0.5 \mu\text{l}$) resulted in more CORT secretion than the lower dose (2.5 $\mu\text{g}/0.5 \mu\text{l}$). 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 CB₁ 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 CB₁ 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 CB₁ 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 CB₁ 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 CB₁ 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 CB₁ 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 CB₁ 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 CB₁ 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 CB₁ receptors are found localized on a subpopulation of inhibitory interneurons (McDonald and Mascagni, 2001), suggesting an important regulatory role for CB₁ receptor transmission within the BLA through endocannabinoid signaling. Several studies have reported strong inhibition of BLA interneurons after application of CB₁ 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 CB₁-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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Exhibit F

STUDY

*Effects of Intra-Amygdala of CB₁ Receptor
Agonists on the Reconsolidation of Fear-
Potentiated Startle*

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2006



LEARNING MEMORY

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 CBI receptor agonists on the reconsolidation of fear-potentiated startle

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The cannabinoid CBI receptor has been shown to be critically involved in the extinction of fear memory. Systemic injection of a CBI 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 CBI 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 CBI receptor antagonist. In contrast, the effect of CBI agonists on reconsolidation was no longer seen if memory reactivation was omitted. Concomitant with block of reconsolidation, CBI 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 CBI 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 (Brodtkin 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 anandamide 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 μ g 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 $171.4\% \pm 8.3\%$ ($n = 6$) in vehicle controls, $99.0\% \pm 13.6\%$ (1 μ g per side, $n = 5$), and $46.0\% \pm 7.7\%$ (11 μ g 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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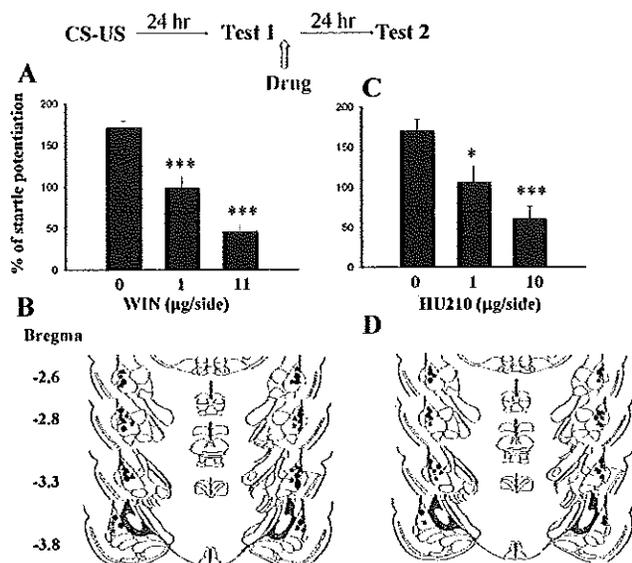


Figure 1. CB1 receptor agonists block reconsolidation of fear memory. (A) Rats were infused with vehicle ($n = 6$), 1 μg of WIN ($n = 5$), or 11 μg of WIN ($n = 10$) within 1 h after the test, and memory retention was assessed 24 h later. *** $P < 0.001$ vs. vehicle. (B) Cannula tip placements from rats 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 $170.5\% \pm 14.1\%$ ($n = 6$) in vehicle controls, $106.4\% \pm 19.9\%$ (1 μg per side, $n = 5$, $P < 0.05$ vs. vehicle), and $61.1\% \pm 15.2\%$ (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.

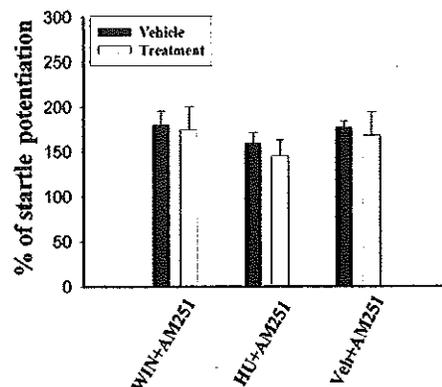


Figure 2. Block of the effect of CB1 agonists on reconsolidation by AM251. AM251 (20 μg per side) was administered 20–25 min before WIN (11 μg per side) or HU210 (10 μg per side). There was no difference in the amount of startle amplitude between the vehicle and WIN/AM251 groups ($t_{(12)} = 0.18$, $P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

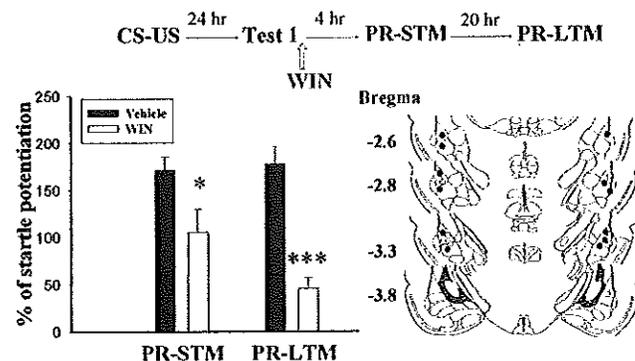


Figure 3. Effects of post-Test 1 infusion of WIN on STM and LTM. Rats were infused with vehicle or 11 μg of WIN within 1 h after the test, and STM was assessed at 4 h and followed by LTM at 24 h after administration of WIN. * $P < 0.05$, *** $P < 0.001$ vs. vehicle. Cannula tip placements from rats infused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

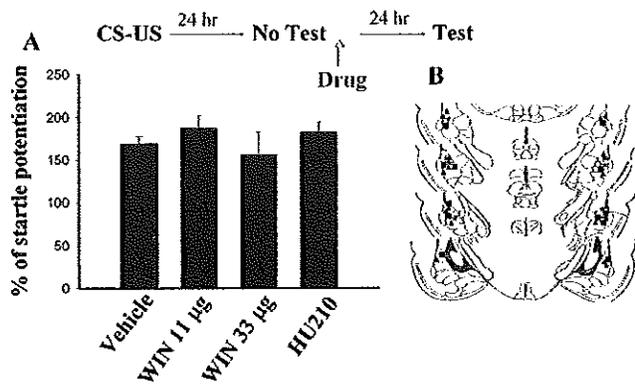


Figure 4. Requirement of memory retrieval for the action of CB1 agonists. (A) There was no difference in startle reflex between vehicle- and WIN- or HU210-treated rats when Test 1 was omitted. (B) Cannula tip placements from rats infused with vehicle (▲), 11 µg WIN (□), 33 µg WIN (■), or HU210 (★).

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

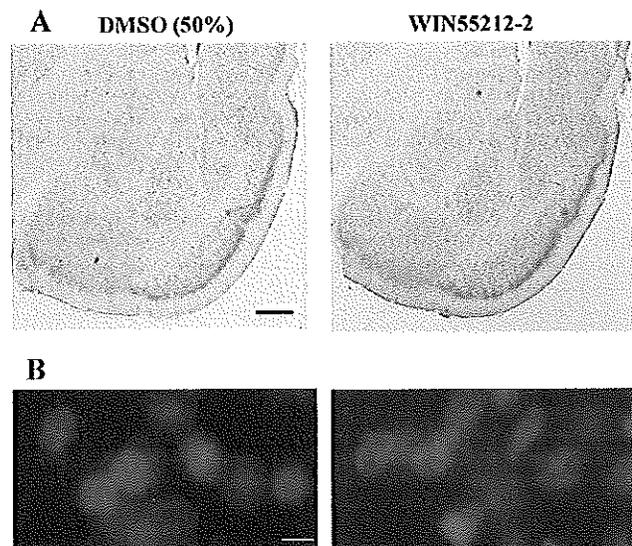


Figure 5. WIN55212-2 did not lesion the amygdala. (A) Representative photomicrographs show amygdala slices from rats infused with DMSO (left) or WIN (right). There was no evidence of increased cell loss or gliosis in the amygdala in the DMSO or WIN-treated animals. Bar, 0.5 mm. (B) WIN (11 µg/side) or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

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 $151.7\% \pm 18.3\%$ ($t_{(4)} = 5.50$, $P < 0.01$ 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.

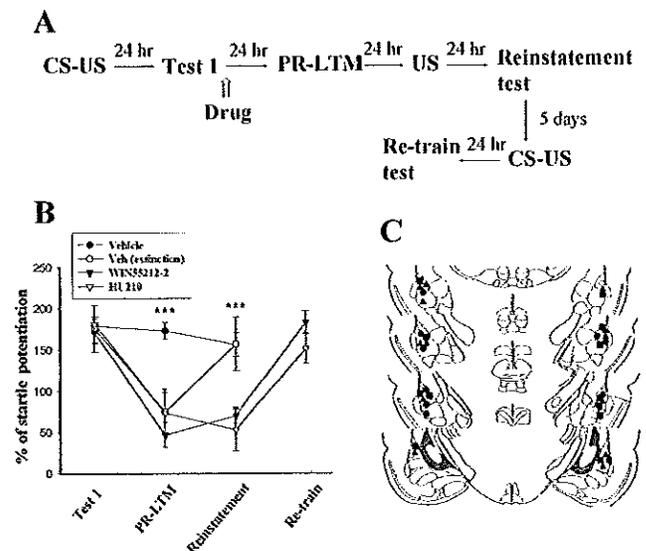


Figure 6. Retardation of reinstatement of fear memory by CB1 receptor agonists. (A) Behavioral procedure used for the experiments shown in B. (B) WIN (11 µg per side) or HU210 (10 µg per side) were infused into the amygdala bilaterally within 1 h after Test 1, which blocked reconsolidation. Amnesia resulting 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.

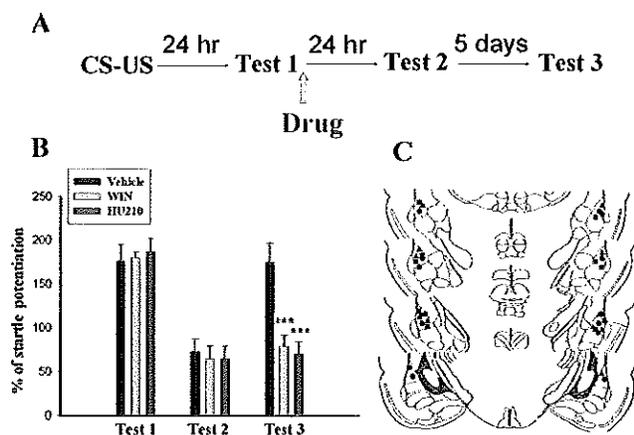


Figure 7. Retardation of spontaneous recovery by CB1 receptor agonists. (A) Behavioral procedure used in the experiment shown in B. (B) Animals were trained and then tested the next day. WIN (11 μ g per side) or HU210 (10 μ g per side) were infused into the amygdala bilaterally within 1 h after the test. 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 (\bullet), WIN (\blacktriangle), or HU210 (\blacksquare) 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 spe-

cific 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-

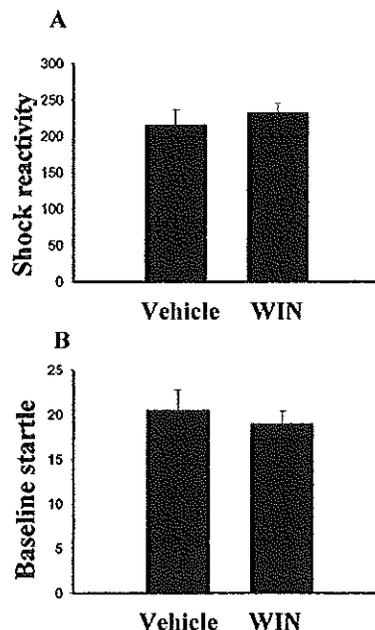


Figure 8. Effect of WIN on shock reactivity and baseline anxiety. Conditioned rats received an intra-amygdala infusion of WIN (11 μ g/side, $n = 5$) and 30 min later were presented with three shocks and 42 startle stimuli (0.6-mA, 0.5-sec shocks, 95-dB noise-burst startle). 3 d later, the same rats were 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 μ L at a rate of 0.1 μ L/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)] $\times 100$.

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- μ m thickness) were stained for Nissl bodies and DNA dye Hoechst 33,342 (bis-benzimidazole, 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 \pm SEM.

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