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# Anandamide Enhances Extracellular Levels of Adenosine and Induces Sleep: An In Vivo Microdialysis Study

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**Study Objectives:** The principal component of marijuana, delta-9-tetrahydrocannabinol increases sleep in humans. Endogenous cannabinoids, such as N-arachidonoylethanolamine (anandamide), also increase sleep. However, the mechanism by which these molecules promote sleep is not known but might involve a sleep-inducing molecule such as adenosine. Microdialysis samples were collected from the basal forebrain in order to detect levels of adenosine before and after injection of anandamide.

**Design:** Rats were implanted for sleep studies, and a cannula was placed in the basal forebrain to collect microdialysis samples. Samples were analyzed using high-performance liquid chromatography.

**Settings:** Basic neuroscience research laboratory.

**Participants and Interventions:** Three-month-old male F344 rats. At the start of the lights-on period, animals received systemic injections of dimethyl sulfoxide (vehicle), anandamide, SR141716A (cannabinoid receptor 1 [CB1] antagonist), or SR141716A and anandamide. One hour after injections, microdialysis samples were collected (5μL) from the basal forebrain every hour over a 20-minute period for 5 hours. The samples were immediately analyzed via high-performance liquid chromatography for adenosine levels. Sleep was also recorded continuously over the same period.

**Measurements and Results:** Anandamide increased adenosine levels compared to vehicle controls with the peak levels being reached during the third hour after drug injection. There was a significant increase in slow-wave sleep during the third hour. The induction in sleep and the rise in adenosine were blocked by the CB1-receptor antagonist, SR141716A.

**Conclusions:** Anandamide increased adenosine levels in the basal forebrain and also increased sleep. The soporific effects of anandamide were mediated by the CB1 receptor, since the effects were blocked by the CB1-receptor antagonist. These findings identify a potential therapeutic use of endocannabinoids to induce sleep in conditions where sleep may be severely attenuated.

**Key Words:** Sleep, anandamide, adenosine, cannabinoids, insomnia, microdialysis

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## INTRODUCTION

MARIJUANA IS A SPANISH WORD OF MEXICAN ORIGIN FOR THE LEAVES, FLOWERING TOPS, AND OTHER MATERIAL FROM THE PLANT, *CANNABIS SATIVA*. Marijuana has been used for hundreds of years for mystical and religious ceremonies, for social interaction, and for therapeutic uses. The primary active ingredient in marijuana is delta-9 tetrahydrocannabinol ($\Delta^9$-THC),[1] one of some 60 21-carbon terpenophenolic compounds knows as cannabinols, which exerts its actions via 2 cannabinoid receptors referred to as $CB_1$ and $CB_2$ receptors.

Endogenous cannabinoids have been isolated from peripheral and nervous tissue. Among these, N-arachidonoylethanolamine (anandamide, AEA) and 2-arachydonoylglycerol are the 2 best-studied examples.[2-5] Behaviorally, AEA increases food intake[6-8] and induces hypomotility[9] and analgesia.[10] Anandamide also induces sleep in rats.[11] Cannabinoid stimulation stabilizes respiration by potently suppressing sleep apnea in all sleep stages.[12] In humans, marijuana and $\Delta^9$-THC increase stage 4, or deep, sleep.[13-18] The mechanism by which the cannabinoids induce sleep is not known, hampering the development of this drug for possible therapeutic use.

The sleep-inducing effects of cannabinoids could be linked to endogenous sleep factors, such as adenosine (AD). There is substantial evidence that AD acts as an endogenous sleep factor. Systemic or intracerebroventricular administration of AD promotes sleep.[19,20] Extracellular levels of AD measured by microdialysis are higher in spontaneous waking than in sleep in the basal forebrain and not in other brain areas such as thalamus or cortex.[21,22]

Given this evidence, we hypothesized that the soporific effects of AEA could be associated with increased AD levels. In the present study, extracellular AD levels were assessed in the basal forebrain via the microdialysis method. The basal forebrain was sampled because this region is particularly sensitive to AD.[22] The cholinergic neurons located in the basal forebrain are implicated in maintaining waking behavior, and it is hypothesized that sleep results from the accumulating AD, which then inhibits the activity of the wake-active cholinergic neurons.[21] Our results show that systemic application of AEA leads to increased AD levels in the basal forebrain during the first 3 hours after injection, and total sleep time is increased in the third hour. These findings identify a possible mechanism by which the endocannabinoid system influences sleep.

## METHODS

### Animals

Young (3-month-old) male F344 rats (N = 6) (National Institutes of Aging, USA) were housed at constant temperature (21°C ± 1°C) under controlled light-dark cycle with lights on from 7:00 AM to 7:00 PM. Food and water were provided ad libitum.

### Operations

Animals were implanted under anesthesia (acepromazine [0.75 mg/kg], xylazine [2.5 mg/kg], and ketamine [22 mg/kg] administered intramuscularly) with cortical sleep-recording electrodes. These consist-

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ed of 4 stainless-steel screws fixed to the parietal and occipital bones through trepanations and were used to record the electroencephalogram (EEG). Two miniature screws were inserted 2 mm on either side of the sagittal sinus and 3 mm anterior to bregma (frontal cortex). The other two screws were located 3 mm on either side of the sagittal sinus and 6 mm behind bregma (occipital cortex). The EEG was recorded from two contralateral screws (frontal-occipital). Two stainless-steel multiwire electrodes inserted into the nuchal muscles were used to monitor the electromyogram (EMG).

All electrodes were secured onto the skull using dental cement. In each animal, a guide cannula (IC guide. BioAnalytical Systems [BAS], West Lafayette, Ind,) was placed stereotaxically [23] into the basal forebrain (A = -.35; L = 2.0; H = -7.5). The guide cannula was covered with a thin layer of acrylic cement.

Animals were allowed to recover for at least seven days after the operation. During this period, they were attached to EEG recording leads to habituate them to the recording conditions. All procedures were in accordance with the American Association for Accreditation of Laboratory Animal Care policy on care and use of laboratory animals.

### Drugs

Anandamide was synthesized in our lab as previously described and tested for identity and chemical purity (> 98%),[10,24] and dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co (St. Louis, MO, USA). The SR141716A was obtained from Sanofi (Paris, France). All compounds were dissolved in DMSO (40% in saline). All drugs were given intraperitoneally because the DMSO that was used to dissolve the AEA clogs the microdialysis membrane, thereby making direct brain injections unfeasible.

### Microdialysis Sampling Procedures

A microdialysis probe (BAS: 1 mm in length, polyacrylonitrile, molecular weight cut off = 30,000 d; 340 μm outside diameter) was inserted through the guide cannula into the target structure at 7:00 AM. We determined the amount of time needed for extracellular levels of AD to stabilize after probe insertion. It took about 6 to 7 hours after probe insertion for the AD levels to stabilize. Artificial cerebrospinal fluid was perfused at a flow rate of 0.25 μL per minute. Samples were collected from the outlet tubing (FEP Teflon tubing: 0.65 mm outside diameter x 0.12 mm internal diameter, BAS, West Lafayette, Ind) every 20 minutes.

The next day, the animals received DMSO as a vehicle (40% in saline, 1 mL intraperitoneally at 7:00 AM) and sleep recordings were obtained for 5 hours (7:00 AM - 12:00 noon). The following day, AEA (10 mg/kg, intraperitoneally, in 1 mL) was administered, and sleep was recorded for 5 hours. The AEA was administered during the rat's normal sleep time so as to be consistent with previous studies.[11,25] Twenty-four hours after ANA injection, SR141716A was administered to all rats at 1 mg/kg (intraperitoneally in 1 mL). To block the effects of AEA, 24 hours after the previous manipulation, all animals received SR141716A first (1 mg/kg in 0.5 mL), and 15 minutes later, AEA was injected (10 mg/kg in 0.5 mL). All rats received all pharmacologic treatments on different days at the same hour (7:00 AM). Immediately after the injections, the rats were returned to their recording cages, and sleep was recorded. One hour after injection, microdialysate samples (5 μL) were collected over a 20-minute period. Thereafter, microdialysis samples were collected over a 20-minute period at the start of each hour, and this continued for the next 4 hours (flow rate = 0.25 μL/minute).

### Neurochemical Analysis of AD

All samples were analyzed with a high-performance liquid chromatography (BAS, West Lafayette, Ind) coupled to BAS UV-8, 254-nm detector and a PM-80 pump (BAS, West Lafayette, Ind). The mobile phase consisted of 10-mmol $NaH_2PO_4$ (pH 4.5) plus 9% of $CH_3OH$. Separation was achieved by a microbore column (biophase octyl, 5 μm, 250 x 4.6 mm, BAS, West Lafayette, Ind) attached to the injector (CC-5e) and to the detector (UV-116A, BAS, West Lafayette, Indiana, USA). Chromatographic data were recorded on a personal computer, and peak heights of AD in microdialysis samples were compared to standards using the chromatography report software (BAS, West Lafayette, Indiana, USA).

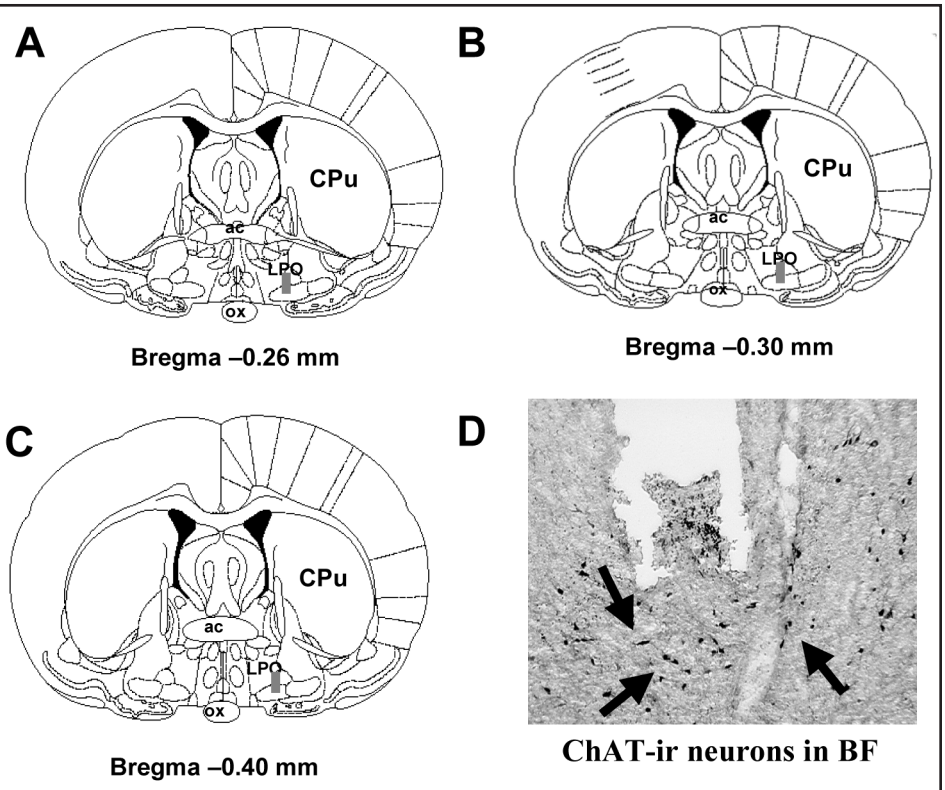


**Figure 1**—Schematic representation of localization of the membrane probe in the basal forebrain. Stereotaxic coordinates, drawings, and abbreviations are based on the Paxinos and Watson rat atlas. Photomicrograph of the track of the microdialysis probe in the magnocellular preoptic area (D). Immunohistochemistry for choline acetyltransferase (ChAT), the rate-limiting enzyme in the synthesis of acetylcholine, shows the presence of cholinergic neurons surrounding the probe. ac refers to anterior commisure; CPu, caudate putamen; LPO, lateral preoptic area; ox, optic chiasm.

### Analyses of Sleep Recordings

Contralateral frontal-occipital EEG screw electrodes were used for EEG acquisition. The EEG data were filtered at 70 Hz (low-pass filter) and 0.3 Hz (high-pass filter) using a Grass electroencephalograph (Astro-Med, Inc., West Warwick, Rhode Island, USA) and continuously sampled at 128 Hz. The EEG data recordings were scored manually on a computer (Icelus software; Mark Opp, Univ Michigan, Ann Arbor, MI, USA) in 12-second epochs for wakefulness (W), slow-wave sleep (SWS), and rapid-eye-movement (REM) sleep. The W state was identified by the presence of desynchronized EEG and high EMG activity. The SWS consisted of high-amplitude slow waves together with a low EMG tone relative to awake. The REM sleep was identified by the presence of regular theta activity coupled with low EMG relative to SWS.

### Histologic Verification of Probe Location

Animals were sacrificed after experiments with a lethal dose of pentobarbital and perfused transcardially with saline followed by 4%

formaldehyde. The brain was removed and postfixed overnight in 4% formaldehyde followed by 20% sucrose 0.1-mol phosphate-buffered saline for 48 hours. The brains were cut (frozen sections, 30 μm, coronal), and tissue sections lightly processed for visualization of choline acetyltransferase (ChAT). In this procedure, the tissue sections were incubated overnight in a rabbit anti-ChAT primary antibody (1:10,000; Chemicon, Temecula, CA, USA). The next day, after washes, the tissue was exposed to the secondary antibody (for 1 hour) followed by incubation in the avidin-biotin complex (for 1 hour, Vector, Burlingame, CA, USA). The antigen was visualized using the diaminobenzidine method. The sections were then mounted onto slides and lightly stained with Neutral Red stain. The section was then dehydrated in graded alcohol solutions and then cover slipped. The cannula was localized using Paxinos and Watson's rat atlas.

### Statistical Analysis

The statistical analysis for the natural sleep-cycle data as well as AD-concentration values was performed using a 1-way repeated measures analysis of variance (ANOVA) (STATVIEW, SAS Institute, Inc., Cary, NC). When a significance of $P < .05$ was found, the posthoc Sheffe test determined individual group differences ($P < .05$).

## RESULTS

### Adenosine Levels After AEA Administration

Probes were placed in the basal forebrain in an area containing cholinergic neurons (Figure 1). Chromatograms showed that AD peak appeared after 6 minutes of injection into the high-performance liquid chromatograph (data not shown). A repeated-measures ANOVA of AD levels across the 5-hour recording period found no significant differences (hour effect) after DMSO injections (Figure 2A). Extracellular levels of AD increased during the first 3 hours after administration of AEA (Figure 2A). The highest levels of AD were reached at the end of the third hour (mean ± SEM nmol of AD [DMSO = 108 ± 14.2 vs AEA = 235.0 ± 4.7; $P < .0001$]). In the fourth hour, AD levels decreased significantly relative to the third hour (mean ± SEM nmol of AD [DMSO = 117.0 ± 14.2 vs AEA = 75.2 ± 8.2; $P < .0001$]) and to vehicle controls. Five hours after the administration of AEA, AD levels were not significantly different from controls (Figure 2A).

Administration of the $CB_1$-receptor antagonist, SR141716A, significantly decreased AD levels in the second (mean ± SEM nmol of AD [DMSO = 75.5 ± 3.4 vs SR141716A = 11.86 ± 8.2; $P < .0001$]), third, and fourth hour after injection. When SR141716A was injected before AEA, it blocked the increase in AD in the first 3 hours (mean ± SEM nmol of AD [AEA = 154.0 ± 17.3 vs SR141716A+ AEA = 18.53 ± 9.2; $P < .0001$]), and produced a decrease in AD in the fourth hour (Figure 2A).

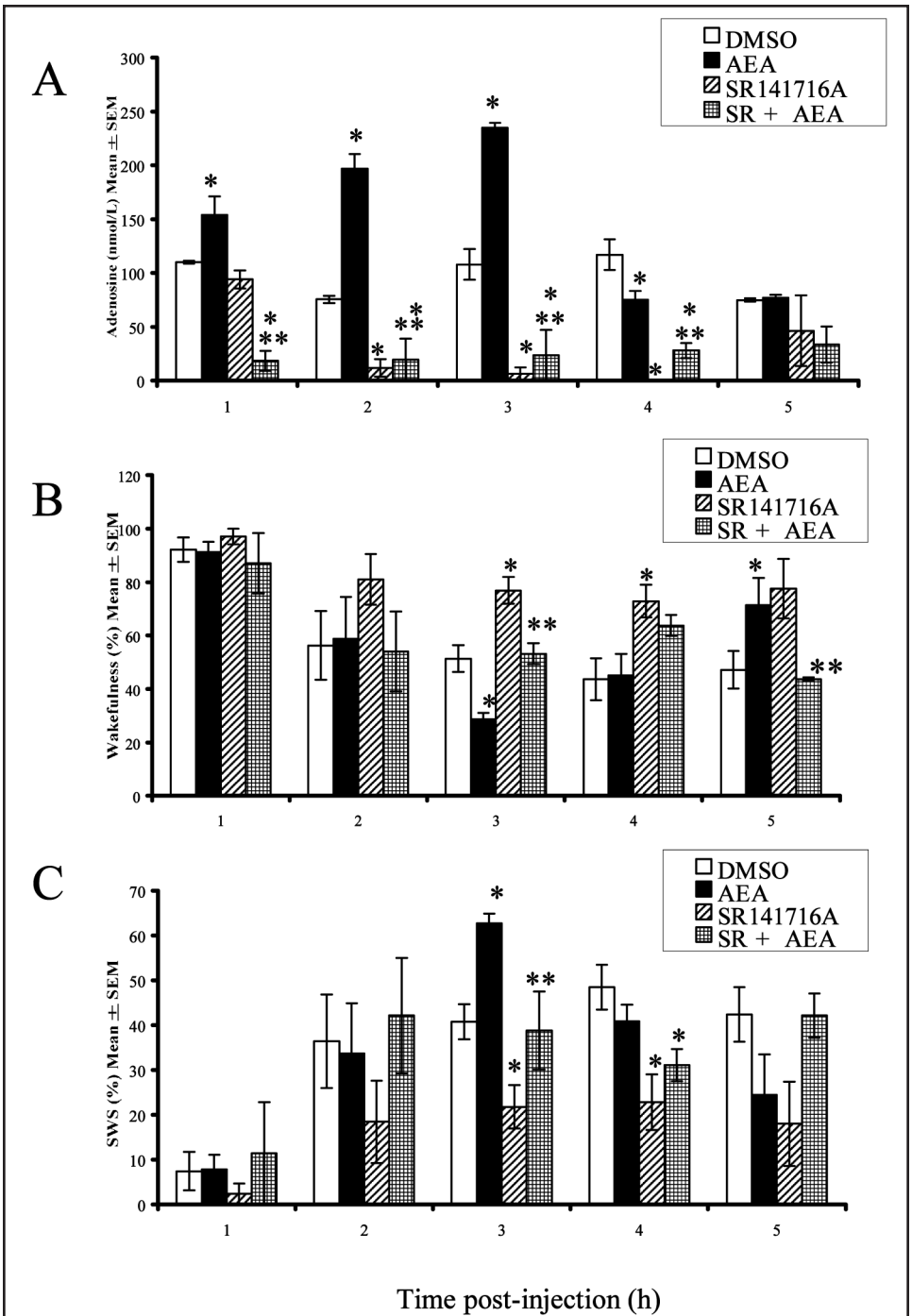


**Figure 2**—(A) Extracellular levels of adenosine measured in basal forebrain during a 5-hour period after administration of DMSO (dimethyl sulfoxide), anandamide (AEA), SR141716A, or SR141716A + AEA . Percentage of wakefulness (B) and slow-wave sleep (SWS)(C) during the 5-hour period after systemic injection of drugs. Note the association between adenosine, wakefulness, and SWS with AEA . Values are mean ± SEM (N = 6). * vs DMSO ($P < .05$); ** vs AEA ($P < .05$).

### Analysis of Sleep After AEA Administration

The percentage of time spent in W, SWS, and REM sleep was determined at the end of each hour. There was a decrease in W and an increase in SWS with the vehicle (DMSO) during hours 2 to 5 after the injection relative to the first hour (Figure 2B and 2C). However, this represents the increase in sleep time that normally occurs in nocturnal rodents during the first half of the lights-on period.

Injection of AEA (10 mg/kg, intraperitoneally) caused a decrease in W and a significant increase in SWS time in the third hour (54.5%), compared to vehicle (Figure 2C). Total sleep time increased because of an increase in SWS (mean percentage ± SEM [DMSO = 40.70 ± 3.9; AEA = 62.70 ± 2.2; $P < .001$]). Rapid-eye-movement sleep was not different compared to vehicle (Data not shown). The decrease in W and increase in SWS obtained after AEA injection was consistent with our previous studies.[11,25]

In agreement with a previous report,[26] animals that received SR141716A (1 mg/kg, intraperitoneally) were awake more during the third hour after injection (mean percentage ± SEM [DMSO = 51.34 ± 5.0; SR141716A = 76.86 ± 5.0; $P < .0006$]; Figure 2B) and had decreased SWS (mean percentage ± SEM [DMSO = 40.70 ± 3.9; SR141716A = 21.76 ± 4.8; $P < .001$]; Figure 2C).

Administration of SR141716A plus AEA abolished the increase in SWS caused by AEA alone in the third hour (mean percentage ± SEM [ANA = 62.70 ± 2.2; SR141716A + AEA = 38.76 ± 17.7; $P < 0.001$]; Figure 2C).

We examined the latency of sleep onset and the length and number of SWS bouts, but there was no significant difference between AEA compared with DMSO. Delta power during SWS was also not significantly different between AEA and DMSO, but this might be because of high variability in delta power between animals.

## DISCUSSION

Previous reports have found that application of AEA directly to the brain (via microinjection) increases total sleep time and SWS.[11,25] We have now shown that systemic administration of AEA also has the same effect. More importantly, the increased sleep is associated with increased extracellular levels of AD in the basal forebrain.

The increase in sleep induced by AEA occurred during the third hour after injection of the compound and was associated with peak levels of AD. In each of the first 2 hours, AD levels were significantly higher compared to vehicle injections, with a peak in AD occurring in the third hour. Increased sleep was not evident in the first 2 hours, suggesting that a threshold accumulation of AD might be necessary to drive sleep. In the fourth hour, AD decreased dramatically relative to the third hour, and the levels were not different from those observed after vehicle administration. There was no significant difference in delta power between AEA and DMSO, even though the percentage of SWS was higher with AEA. The high variability between animals in delta power may have obscured an effect.

Sleep is hypothesized to result from accumulating AD levels, and then there is a decline as a result of sleep.[21] This effect is present in the basal forebrain and not in other brain areas.[22] Thus, this purine is hypothesized to act as a homeostatic regulator of sleep; its buildup increases the sleep drive, and as AD levels decline, sleep drive also diminishes. The present data are consistent with this hypothesis in that peak sleep levels occur with peak AD levels, and then as a result of sleep, AD levels also decline (fourth hour in Figure 2B).

The $CB_1$-receptor antagonist blocked the AEA-induced induction of AD as well as the sleep-inducing effect. The $CB_1$-receptor antagonist SR141716A has been tested in diverse behavioral paradigms, and it blocks the effects induced by AEA.[9,11] Santucci and coworkers demonstrated that administration of SR141716A increases W and decreases SWS.[26] Here we replicated these effects but also demonstrated that the increase in AD levels after injection of AEA were blocked by the $CB_1$-receptor antagonist.

The AEA exerts its effect via the $CB_1$ receptor and hyperpolarizes the neuron.[27] The $CB_1$ receptors are coupled to the $G_i/G_o$ family of G protein heterotrimers. Activation of the $CB_1$ receptor inhibits adenylate cyclase and decreases synthesis of cAMP.[5,28] In rats, the $CB_1$ receptor is localized in the cortex, cerebellum, hippocampus, striatum, thalamus, and brainstem.[29,30] The $CB_1$ receptor is also present on basal-forebrain cholinergic neurons as determined by immunocytochemistry.[31] The $CB_1$-receptor mRNA is present in the basal forebrain.[32,33] This receptor is also present in the brainstem where the cholinergic pedunculopontine tegmental region is implicated in W.[34] Microinjection of AEA into this region decreases W and increases REM sleep.[11]

The $CB_1$ receptors are also localized in the thalamus,[35] an area implicated in producing slow waves in the EEG.[34] Activation of these receptors in the pedunculopontine tegmentum, basal forebrain, and thalamus may decrease the firing of wake-active neurons, resulting in sleep. Additionally, accumulation of AD in the basal forebrain may inhibit the cholinergic neurons[21] and also increase sleep. Direct injections of the AEA into the basal forebrain were not possible since AEA dissolves only in DMSO and alcohol. Moreover, delivery of the AEA dissolved in DMSO clogs the microdialysis membrane.

The mechanism by which AEA increases AD in the basal forebrain is not known, even though both AD and AEA could directly inhibit the wake-active neurons given the inhibitory action of these agents on their receptors. Nevertheless, there is evidence of an interaction between the adenosinergic and the endocannabinoid systems.[36] For example, the motor impairment induced by the principal component of cannabis, $\Delta^9$-THC, is enhanced by adenosine $A_1$-receptor agonists.[37] We now show that stimulation of the endocannabinoid system via the $CB_1$ receptor increases AD in the basal forebrain. The endocannabinoids and AD may regulate sleep homeostasis via second and third messengers, as we have hypothesized.[38]

Previously, investigators have shown that stage 4 sleep (deep sleep) in humans is increased in response to administration of $\Delta^9$-THC or smoking of marijuana cigarettes.[15,16] We now show that such a soporific effect is associated with an increase in AD levels in the basal forebrain. Cannabinoid stimulation suppresses sleep apnea in rats,[39] and $A_1$-receptor stimulation also has the same effect.[12] The endocannabinoid system also influences other neurotransmitter systems,[40-42] in particular, inhibiting the glutamatergic system.[43] It would be important to determine whether endogenous levels of specific neurotransmitter systems are changed as a result of AEA-induced activation of the $CB_1$ receptor. Irrespective of the mechanism involved, our studies underscore the importance of endocannabinoid-AD interactions in sleep induction and open new perspectives for the development of soporific medications.


## ACKNOWLEDGMENTS

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## ABBREVIATIONS

N-arachidonoylethanolamine (anandamide, AEA)
[N-(piperidine-1-yl)-s-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] hydrochloride (SR141716A)
adenosine (AD)
choline acetyltransferase (ChAT)
dimethyl sulfoxide (DMSO)
electroencephalogram (EEG)
electromyogram (EMG).
rapid eye movement (REM) sleep
slow wave sleep (SWS)
wakefulness (W)

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