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The Nonpsychoactive Cannabis Constituent Cannabidiol Is a Wake-Inducing Agent

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Cannabidiol (CBD) is a constituent of *Cannabis sativa* that induces nonpsychotropic effects, and some of its biological actions in sleep have been described by the authors' group. Here, the authors report that when administered 10 or 20 μg/1 μl during the lights-on period directly into either lateral hypothalamus (LH) or dorsal raphe nuclei (DRN), which are wake-inducing brain areas, CBD enhanced wakefulness and decreased slow wave sleep and REM sleep. Furthermore, CBD increased alpha and theta power spectra but diminished delta power spectra. Additionally, CBD increased c-Fos expression in LH or DRN. These findings suggest that this cannabinoid is a wake-inducing compound that presumably activates neurons in LH and DRN.

Keywords: wakefulness, delta power spectra, cannabinoid receptor, insomnia, slow wave sleep

Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) are two major constituents of *Cannabis sativa* (Mechoulam, Shani, Edery, & Grunfeld, 1970). In addition, Δ^9-THC is a psychoactive compound and produces stereotypical behaviors (Adams & Martin, 1996) activating the CB_1 receptor, which is thought to be responsible for the majority of the effects in the central nervous system (Ameri, 1999). Despite CBD's binding to the CB_1 receptor (Thomas et al., 2007), this molecule is not psychoactive. Thus, the pharmacological and pharmaceutical interest in this compound has risen (Klein & Newton, 2007), leading to investigations of the biochemistry of this molecule (McGilveray, 2005; Mechoulam & Hanus, 2002).

Some of its pharmacological effects have been reported (Dirikoc, Priola, Marella, Zsürger, & Chabry, 2007; Esposito et al., 2007; Fadda, Robinson, Fratta, Pertwee, & Riedel, 2004; Mechoulam, Peters, Murillo-Rodriguez, & Hanus, 2007). For example, on one hand CBD presents anticonvulsant, sedative, and anxiolytic properties (Chesher, Jackson, & Malor, 1975; Cortesi & Fusar-Poli, 2007; Moreira, Aguiar, & Guimaraes, 2006; Pickens, 1981; Russo & Guy, 2006). Furthermore, Guimaraes, Chiaretti, Graeff, and Zuardi (1990) have found that administration of CBD (2.5 mg/kg–10 mg/kg) induces an anxiolytic-like effect as evaluated by the elevated plus maze assay, whereas it displays neuroprotective properties against 6-hydroxydopamine toxicity (Lastres-Becker, Molina-Holgado, Ramos, Mechoulam, & Fernández-Ruiz, 2005).

On the other hand, several studies have demonstrated that the administration of Δ^9-THC promotes sleep (Feinberg, Jones, Walker, Cavness, & Floyd, 1976; Feinberg, Jones, Walker, Cavness, & March, 1975), whereas CBD induces contradictory results. For instance, Monti (1977) showed a reduction in sleep, and later Carlini and Cunha (1981) reported an improvement of sleep in insomniacs. The methodological differences among these studies might be the reason for these contradictory results. Just recently, it was demonstrated in humans that CBD increased wakefulness (Nicholson, Turner, Stone, & Robson, 2004). In line with these findings, our group has reported that icv injections of CBD in rats promoted wakefulness and decreased slow wave sleep (SWS) and REM sleep (REMS; Murillo-Rodríguez, Millán-Aldaco, Palomero-Rivero, Mechoulam, & Drucker-Colín, 2006). If CBD increases wakefulness, one would expect that the administration of this compound into wake-inducing nuclei, such as the lateral hypothalamus (LH) or dorsal raphe nuclei (DRN), would elicit an enhancement in alertness. However, to date this question has not been fully examined. Here, we investigated whether the direct microinjection of CBD into either LH or DRN increases wakefulness.

Method

Subjects

Male Wistar rats (N = 48; 250 g–300 g) were housed at a constant temperature (21 ± 1 °C) on a controlled light–dark cycle

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(lights on from 07:00 to 19:00). All experimental procedures were conducted in accordance with standards of animal care approved by the local Institutional Care and Use Committees and followed the Guidelines for the Care and Use of Mammals in Neuroscience, guidelines released by the Mexican Institutes of Health Research (DOF.NOM-062-Z00-1999), and the National Institutes of Health (1986) *Guide for the Care and Use of Laboratory Animals.* All efforts were made to minimize animal suffering and to reduce the number of rats used.

Surgery

Under aseptic conditions and deep anesthesia (acepromazine [0.75 mg/kg], xylazine [2.5 mg/kg], and ketamine [22 mg/kg ip]), all rats were implanted with electrodes to record electroencephalogram (EEG) and electromyogram (EMG) and with a unilateral cannula (23-gauge) aimed into either the LH (bregma: A = −3.3; L = +1.6; H = −8.2; Paxinos & Watson, 1986/1998) or DRN (bregma: A = −7.8; L = +0.2; H = −7.1; Paxinos & Watson, 1998). Right after the surgeries, rats were placed singly into the sleep-recording chambers for habituation (~7 days). Throughout the experiment, rats had food and water available ad libitum, and they lived under a constant temperature (21 ± 1 °C) and on a controlled light–dark cycle (lights on from 07:00 to 19:00). All procedures have been previously reported by our group (Murillo-Rodríguez et al., 2006; Murillo-Rodríguez, Vázquez, Millán-Aldaco, Palomero-Rivera, & Drucker-Colín, 2007).

Drugs

CBD was synthesized in our laboratory as described previously (Mechoulam, Parker, & Gallily, 2002), and it was dissolved in vehicle (PEG/saline; 5:95 vol/vol).

Pharmacological administrations. On the test day, all rats received randomly at the beginning of the lights-on period the following treatments: Vehicle (VEH, $n = 6$), CBD (10 μg/1 μl, $n = 6$), or CBD (20 μg/1 μl, $n = 6$). An additional group (sham, $n = 6$) was included to determine whether the injection of VEH would modify the sleep of the rats. The administrations were done at 07:00, and drugs were injected manually using a 5-μl Hamilton microsyringe (Hamilton Co., Reno, NV) attached via tubing (FEP Teflon tubing: 0.65-mm o.d. × 0.12-mm i.d.; BAS, West Lafayette, IN) to an injector. The microinjections were done slowly (flow rate: 1 μl/min), and right after the administrations all rats were attached again to the sleep-recording system. We collected and analyzed the sleep–wake recordings from 4-hr postinjection of CBD based on our previous report (Murillo-Rodríguez et al., 2006).

Analyses of sleep recordings. The EEG/EMG signals were recorded using a Grass Model 12 polygraph, filtered (EEG = 0.3–30.0 Hz, EMG = 0.3–1.0 kHz), digitized (sampling frequency = 128 Hz; National Instruments, Austin, TX), and stored on a computer with the aid of an acquisition software (ICELUS, Mark Opp, University of Michigan, Ann Arbor, MI). The EEG and EMG data were scored in 12-s epochs for wakefulness, SWS, and REMS with the aid of a sleep-scoring program (ICELUS, Mark Opp, University of Michigan, Ann Arbor, MI) and classified visually as reported previously (Murillo-Rodríguez et al., 2006, 2007).

To assess whether the effects of CBD on sleep were also on sleep quality, EEG power spectra fast Fourier transformations were performed on artifact-free EEG signals and generated absolute power values for delta (during SWS), theta (during REMS), and alpha (during wakefulness) frequency bands ($\delta = 0.5–4.0$ Hz, $\theta = 6.0–9.0$ Hz, and $\alpha = 12–14$ Hz, respectively). Data of absolute power represent averages obtained across 4 consecutive hours at every time point (12 s) recorded during the experiment. Sleep scoring and all power analyses were carried out by a single examiner unaware of the rat's treatment group.

Immunohistochemical studies. Two days after the end of the sleep studies, all rats received either vehicle ($n = 6$) or CBD (20 μg/1 μl; $n = 6$). One hour after drug injection, all rats were deeply anesthetized with pentobarbital (150 mg/kg ip) and perfused. Rats were decapitated, and brains were removed and dissected (30 μm) on coronal sections that were stained for Fos as previously described (Murillo-Rodríguez et al., 2007). One person unaware of the experimental conditions counted all of the immunoreactive somata at an area adjacent to the injection site (control = 6; CBD = 6) using a 10-square grid (number of somata per unit of slice on a 50-μm square). Sections were included as follows: for LH, between −1.30 and −4.80 mm, and for DRN, between −7.40 and −8.30 mm. All coordinates referred to bregma (Paxinos & Watson, 1998/1986).

Statistical analysis. The data were expressed as $M \pm SEM$, and the comparison between multiple groups were performed by one-way analysis of variance followed by Scheffé's post hoc test, whereas a Student's t test was used for the c-Fos immunoreactive cell quantification (STATVIEW). A $p < .05$ was considered statistically significant.

Results

Figure 1A shows no statistical differences among sham and control (VEH) groups in wakefulness, SWS, and REMS. Additionally, CBD induced a dose-dependent enhancement in the total time of wakefulness ($p < .0001$) and diminished SWS ($p < .0001$) and REMS ($p < .006$).

To directly test whether wake-induced properties of CBD after its injection into LH influence EEG power spectra, we assessed the analysis of the pharmacological properties of this cannabinoid on EEG delta, theta, and alpha power spectra. Compared with the sham and VEH group (control groups), CBD diminished delta ($p < .0001$) and theta ($p < .0001$) power spectras, whereas it increased alpha power spectra ($p < .0001$; Figures 1B, 1C, and 1D, respectively).

These analyses assessed the relative effects of the intrahypothalamic administration of CBD on sleep but did not address its pharmacological properties when injected into DRN. Therefore, we analyzed the effects on sleep of CBD administration (10 or 20 μg/1 μl) into DRN.

The consistent and statistical differences on sleep between CBD-treated and vehicle/sham groups are shown in Figure 2A. Results showed that microinjection of CBD (10 or 20 μg/1 μl) significantly enhanced the total time of waking in a dose-dependent fashion ($p < .0003$), whereas it decreased SWS ($p < .001$) and REMS ($p < .0001$).

Next, the pharmacological effects of CBD on EEG power spectra were assessed. As hypothesized, injection of CBD into DRN

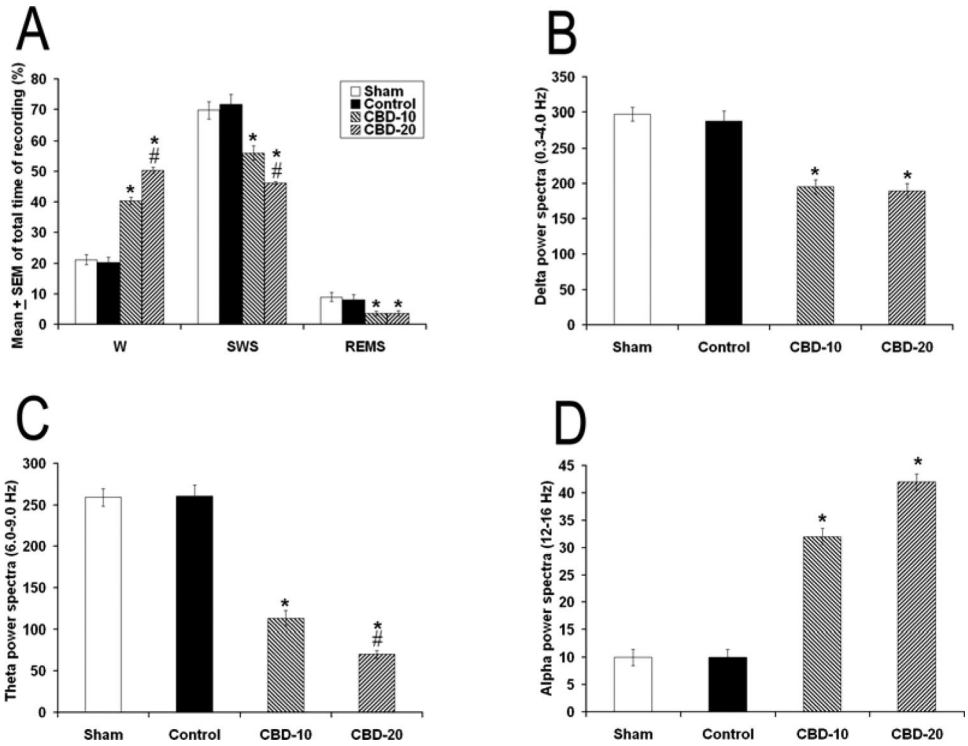

Figure 1. Effects on the total time (4 hr of sleep recordings) of wakefulness (W), slow wave sleep (SWS), and REM sleep (REMS) after the microinjection of either vehicle (control) or cannabidiol (CBD; 10 or 20 μg/1 μl) into lateral hypothalamus at the beginning of the rats' lights-on period (Panel A: *M* ± *SEM* of total time of recording in percentage). CBD decreased delta power spectra during SWS (Panel B) and theta power spectra during REMS (Panel C), whereas it enhanced alpha power spectra during W (Panel D; *M* ± *SEM*; * vs. sham/vehicle, # vs. CBD-10, $p < .05$).

decreased delta ($p < .0004$) and theta ($p < .0001$) power spectra but enhanced alpha power spectra ($p < .0001$; Figures 2B, 2C, and 2D, respectively).

To determine whether the observed behavioral changes after CBD injection into LH were specific to the activity of neurons in that area, we analyzed adjacent sections with antibodies against Fos. Because in the sleep experiments the vehicle used to deliver CBD did not induce statistical differences compared with the sham group, in this part of the study we decided to include only the vehicle group as a control. We found that the highest dose of CBD (20 μg/1 μl) injected directly into the LH increased c-Fos expression (Control, 17.62 ± 0.9 vs. CBD: 42.01 ± 0.3; $p < .005$). Thus, it is reasonable that the wake-inducing effects of CBD after its injection into LH resulted in an induction of *c*-Fos expression in that area. This result might represent the neuroanatomical basis for the wake-inducing properties of CBD because it has been demonstrated that neurons of the hypothalamus are active during wakefulness (Szymusiak et al., 1986).

Complementary data showed that microinjection of the highest dose of CBD into DRN had a statistical significant enhancement of positive staining for Fos (Control, 25.62 ± 3.2 vs. CBD: 48.12 ± 2.8; $p < .005$). We do believe that CBD may induce wakefulness-activating neurons in the DRN because electrophysiological studies have shown a higher firing rate of this nucleus during wakefulness (Urbain, Creamer, & Debonnel, 2006; Wu et al., 2004), which is accompanied by a significant increase in the release of the extracellular levels of 5-HT (Portas & McCarley, 1994).

Discussion

This experiment is the first characterization of the effects on sleep of CBD injected into specific wake-related areas. Our data demonstrate that administrations of CBD into either LH or DRN during the lights-on period increased wakefulness and decreased SWS and REMS in rats. CBD also decreased delta and theta power spectra but increased alpha power spectra.

We adopted an experimental approach to further strengthen the results obtained in the sleep study. We found that the microinjection of CBD into LH induced an expression in the Fos immunoreactive cells. Although the volume injected in the current study could be considered large (1 μl) and it could spread to neighboring areas, the sleep data among the sham and control (VEH) group showed no significant differences. Therefore, it is unlikely that this issue would be responsible for the effects in the current results. In addition, we have previously injected this volume (Méndez-Díaz et al., 2005; Murillo-Rodríguez, Cabeza, Méndez-Díaz, Navarro, & Prospéro-García, 2001; Murillo-Rodríguez, Vázquez-Luis, Millán-Aldaco, Haro, & Drucker-Colín, 2008) and found no effects among control groups.

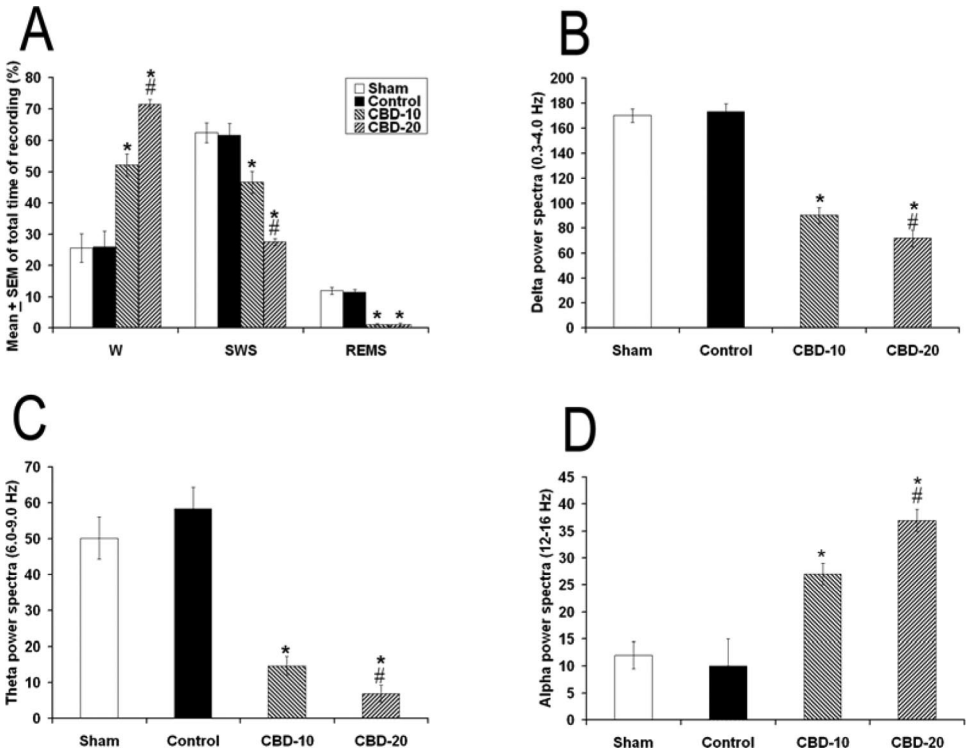

Figure 2. Effects on total time (4 hr of sleep recordings) of wakefulness (W), slow wave sleep (SWS), and REM sleep (REMS) after the administration of either vehicle (control) or cannabidiol (CBD; 10, 20 μg/1 μl) into the dorsal raphe nuclei at the beginning of the rats' lights-on period (Panel A: data represent $M \pm SEM$ of total time of recording in percentages). CBD decreased delta power spectra during SWS (Panel B) and theta power spectra during REMS (Panel C), whereas it enhanced alpha power spectra during W (Panel D; $M \pm SEM$; * vs. sham/vehicle, # vs. CBD-10, $p < .05$).

In the present study, our results suggest that the neuronal mechanism underlying the induction of wakefulness would involve the activity of neurons in the LH. This interpretation is supported by the finding that the activity of the hypothalamus has been associated with wakefulness (Lin, Sakai, Vanni-Mercier, & Jouvet, 1989; Suntsova & Dergacheva, 2003; Szymusiak, Alam, Steininger, & McGinty, 1986). On the basis of this assumption, it is reasonable to suppose that an activation of LH neurons by CBD injections can predict waking.

However, compared with the control groups, a population of neurons in the DRN exhibited c-Fos activity after CBD administration. In line with this, it is known that during waking, the DRN electrophysiological activity is higher (McGinty & Harper, 1976; Trulson & Jacobs, 1979; Urbain et al., 2006), whereas levels of 5-HT are increased (Portas & McCarley, 1994).

For a better understanding of the molecular and neuroanatomical mechanism by which CBD modulates sleep, this subject should be further investigated. Two hypotheses can be drawn from the present data: First, because CBD displays 5-HT agonistic activity (Russo, Burnett, Hall, & Parker, 2005), one might speculate that by enhancing the 5-HT system CBD would modify the sleep. Second, if CBD binds to the CB_1 receptor (Thomas et al., 2007), then it is possible that the activation of this receptor may induce alertness as observed after the administration of the CB_1 antagonist SR141716A (Murillo-Rodríguez, Blanco-Centurión, Sanchez, Piomelli, & Shiromani, 2003; Santucci, Storme, Soubrie, & Le Fur, 1996).

Although the effects of CBD on Fos were quite specific, whether injection of this compound is a primary effect or an effect secondary to the behavioral arousal remains to be described. In this regard, future studies will aim to characterize the neurons activated with double staining with orexin (in the LH) or serotonin (in the DRN). Thus, it may be concluded that although CBD markedly increased wakefulness, this maybe a result of the activation of neurons in LH or DRN.

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