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Acute and subchronic administration of anandamide or oleamide increases REM sleep in rats

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ABSTRACT

Anandamide and oleamide, induce sleep when administered acutely, via the CB1 receptor. Their subchronic administration must be tested to demonstrate the absence of tolerance to this effect, and that the sudden withdrawal of these endocannabinoids (eCBs) does not affect sleep negatively. The sleep–waking cycle of rats was evaluated for 24 h, under the effect of an acute or subchronic administration of eCBs, and during sudden eCBs withdrawal. AM251, a CB1 receptor antagonist (CB1Ra) was utilized to block eCBs effects. Our results indicated that both acute and subchronic administration of eCBs increase REMS. During eCBs withdrawal, rats lack the expression of an abstinence-like syndrome. AM251 was efficacious to prevent REMS increase caused by both acute and subchronic administration of these eCBs, suggesting that this effect is mediated by the CB1 receptor. Our data further support a role of the eCBs in REMS regulation.

1. Introduction

Anandamide (ANA) and oleamide (OLE) are two endogenous molecules with cannabinoid activity that bind to the cannabinoid receptor 1 (CB1) (Axelrod and Felder 1998; Leggett et al., 2004) and are degraded by the fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996). Recently it has been proposed that these lipids are involved in the regulation of several physiological functions and behaviors. For example, core temperature, pain perception, locomotor activity, memory regulation, food intake, sexual behavior, and sleep (Crawley et al., 1993; Basile et al., 1999; Maione et al., 2006; Martínez-González et al., 2004; Rueda-Orozco et al., 2008a, b; Huitrón-Reséndiz et al., 2001).

Regarding anandamide, its acute intracerebroventricular (icv) administration increases non-rapid-eye movement sleep (NREM) and rapid eye movement sleep (REMS) at the expense of waking in rats (Murillo-Rodríguez et al., 1998). Likewise, anandamide administered directly into the peduncle pontine tegmental nucleus (PPTg) causes a similar although stronger effect (Murillo-Rodríguez et al., 2001). This effect was blocked with the CB1 receptor antagonist, SR141716A, indicating that the effect on sleep results from the CB1 receptor activation.

Oleamide was isolated for the first time from the cerebrospinal fluid (CSF) of sleep-deprived cats (Cravatt et al., 1995). We have observed that its systemic administration induces sleep in rats, with shortened sleep latency (Cravatt et al., 1995). In addition, 6 h of sleep deprivation increases 3- to 4-fold the oleamide concentration in rats' CSF. SR141716A, as with anandamide, prevents oleamide's sleep-inducing effects (Mendelson and Basile, 1999). Moreover, SR141716A reduces both NREM and REMS while increasing waking after its systemic administration at a 3 mg/kg dose (Santucci et al., 1996).

Furthermore, mice lacking fatty acid amide hydrolase FAAH (−/−), an enzyme that degrades anandamide and oleamide, exhibit a higher SWS expression and a higher delta power than wild-type mice (Huitrón-Reséndiz et al., 2004), further supporting the notion that eCBs modulate sleep. SR141716A prevents the REMS rebound observed in rats after a 24-h period of selective REMS deprivation (REMSD). During the rebound, consecutive to REMSD, the CB1 receptor increases in the pons of rats (Navarro et al., 2003).

This experimental evidence suggests that anandamide and oleamide improve sleep after their acute administration and this effect is a consequence of mainly, although not entirely, the activation of the CB1 receptor. In this context, we decided to evaluate the effect on sleep of the subchronic administration of these eCBs, during eCBs withdrawal, and after AM251-blocking of the CB1 receptor.

2. Materials and methods

2.1. Subjects

Adult male Wistar rats, weighing 250–280 g, were used. All animals were housed individually in Plexiglas cages. They were maintained at an ambient temperature of 22 ± 1 °C and a controlled

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12:12-h light–dark cycle (08:00 AM–08.00 PM) throughout the study. Food and water were available ad libitum.

2.2. Surgery

Rats ($n=102$) were stereotaxically implanted under anesthesia (cocktail: 66 mg/kg ketamine, 0.26 mg/kg xylazine, and 1.3 mg/kg acepromazine) with a stainless-steel guide cannula (23 gauge) aimed at the lateral ventricle ($P=0.8$, $L=1.5$, $V=3.8$) for icv administration of drugs. Two electrodes were inserted into the hippocampus ($P=4.0$, $L=2.5$, $V=2.5$) according to the Paxinos and Watson atlas (1986) for recording the electroencephalographic (EEG) equivalent from this structure. Although fully develop slow waves are not obtained from this structure, we still have a signal indicative of NREM sleep. In addition, the theta rhythm is easily recorded from the hippocampus, helping us to easily differentiate between waking and REMS. Two additional screw electrodes were implanted into the frontal bones for grounding the animal. Two twisted wire electrodes were placed into the neck musculature for electromyographic (EMG) recordings. Animals were treated according to the Norma Oficial Mexicana (NOM-062-ZOO-199), the Guide for Care and Use of Laboratory Animals established by the National Institutes of Health, and the European Community Council Directive 86/609/ EEC. Additionally, our protocol was approved by the Research and Ethics Committee of the Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM). Every effort was made to minimize the number of animals used and their potential suffering.

2.3. Sleep recording

After surgery, animals were monitored and allowed to recover for 10 days. Upon the completion of this period, rats were habituated to the recording conditions for 24 h. Once the habituation period was completed, rats were divided into different experimental groups. They received a daily icv administration of either vehicle or anandamide or oleamide at 8:00 AM (at the beginning of the light period). Immediately after the administration, the sleep–waking cycle was recorded for 24 h.

The EEG and EMG signals were amplified with a Grass Model 7 polygraph, Amplifier Model 7P511, in a frequency range of 1 to 30 and 30 to 100 Hz, respectively. Signals were acquired and analyzed with the ICELUS® software.

Animals were killed at the end of the experiment with an i.p. overdose of sodium pentobarbital to verify the position of the cannula, Ponceau red stain (5 µl) was injected into the ventricle through the cannula, aimed at dyeing the cerebral ventricle system. Brains were removed and dissected to verify that all the ventricles were stained.

2.4. Chemicals

Arachidonylethanolamide (anandamide, ANA) and oleamide (OLE) were obtained from Sigma Aldrich, and the CB1 antagonist, AM251, was obtained from Cayman Chemical. We decided to use AM251 over SR141716a for two reasons: 1, it is more specific for CB1 receptors and 2, it is commercially available. ANA or OLE was dissolved in a mixture of 5% ethanol in saline, and AM251 was dissolved in 1 µl of dimethyl sulfoxide (DMSO). Doses of drugs and volumes will be specified later in the description of each experiment.

2.5. Experimental protocol

2.5.1. Anandamide and oleamide dose–response curve

To determine the dose at which anandamide or oleamide produce a more reliable effect on sleep, seven groups of animals ($n=6$ for each group) received an acute icv administration. The CONTROL group received 5 µl vehicle (saline with 5% ethanol), ANA1 group (1 µg/5 µl of vehicle), ANA2 (2 µg/5 µl of vehicle), ANA4 (4 µg/5 µl of vehicle), OLE6 (6 µg/5 µl of vehicle), OLE12 (12 µg/5 µl of vehicle) and OLE25 (25 µg/5 µl of vehicle). All experimental drugs were administered at 8:00 AM and sleep was recorded immediately thereafter for 24 h.

2.5.2. Subchronic administration and drug withdrawal

Three subchronic administration groups were used ($n=6$ for each group). The same animals used in groups CONTROL, ANA2 (2 µg/5 µl of vehicle), and OLE25 (25 µg/5 µl of vehicle) for the acute administration were treated daily with identical doses at 8:00 AM for 15 days. These doses were the most efficient in increasing REMS after the acute administration. Rats were not sleep-recorded until day 15 of administration. On this day, immediately after the drug or vehicle administration, animals were sleep-recorded for 24 h. On day 16, all groups received 5 µl vehicle at the same hour and were recorded for 24 h, to detect early withdrawal signs on sleep. In addition, to further document that the sleep-inducing effect of anandamide and oleamide was mediated by the CB1 receptor, an antagonist of this receptor, AM251, was used.

2.5.3. AM251 dose–response curve

We performed an AM251 dose–response curve to determine the effect by blocking the CB1 receptor and, thereby, potentially preventing the activity of the endogenous eCBs. The dose–response curve was started with a dose that was equimolar to 2 µg of anandamide, the very dose producing the most significant effects on sleep by increasing REMS. Four groups were acutely icv administered ($n=6$ for each group), the CONTROL group received 1 µl vehicle (DMSO), AM251-3.2 group (3.2 µg/1 µl of vehicle), AM251-6.4 (6.4 µg/1 µl of vehicle), AM251-12.8 (12.8 µg/1 µl of vehicle). Rats were injected at 8:00 AM and sleep was recorded immediately for 24 h.

2.5.4. Blockade of acute and subchronic administration of anandamide or oleamide

To block the effects of the acute administration of anandamide or oleamide, 3 groups were used ($n=6$ for each group): CONTROL (1 µl of DMSO, 15 min before receiving 4 µl saline with 5% ethanol), AM251/ANA (3.2 µg AM251/1 µl of DMSO, 15 min before receiving 2 µg ANA/4 µl saline with 5% ethanol), and AM251/OLE (3.2 µg AM251/1 µl of DMSO, 15 min before receiving 25 µg OLE/4 µl saline with 5% ethanol). Rats were injected at 8:00 AM and sleep was recorded immediately thereafter for 24 h.

To document the AM251 blocking effect on the subchronic eCBs administration, three additional groups of rats received either the vehicle, anandamide or oleamide during 15 days ($n=6$ for each group) exactly as described for the subchronic administration experiment. On day 15, AM251 was administered as follows: CONTROL (1 µl of DMSO 15 min before administering 4 µl saline with 5% ethanol), AM251/ANA (3.2 µg AM251/1 µl of DMSO, 15 min before administering 2 µg ANA/4 µl saline with 5% ethanol), and AM251/OLE (3.2 µg AM251/1 µl of DMSO, 15 min before administering 25 µg OLE/4 µl saline with 5% ethanol). Rats were injected at 8:00 AM and sleep was recorded immediately thereafter for 24 h.

2.6. Data analysis

Polygraphic recordings were analyzed every 12 s and classified according to the following vigilance stages: wakefulness (W), non-rapid-eye movement (NREM), and rapid eye movement (REMS) sleep. Electrophysiological criteria were used to define these stages of vigilance as follows: W was characterized by the EEG expressing mixed low fast voltage and theta activity, as well as high muscle activity. In NREM, rats showed an EEG with delta waves and EMG with decreased amplitude. Finally, in REMS rats showed an EEG with theta activity and an EMG absent activity (postural atonia). The time spent in W, NREM, and REMS per hour was calculated during two periods of 12 h (total 24 h). Latency of NREM and REM sleep was also calculated by measuring the time

elapsed from the start of the sleep recording to the first NREM bout. REMS latency was considered from the first SWS bout to the first REMS bout. Frequency and average duration of REMS bouts were also calculated.

2.6.1. Statistics

Results of REMS, SWS, and W were compared by a mixed analysis of variance (ANOVA) with a Greenhouse–Geisser correction, and subsidiary ANOVAs to detect changes per hour with an LSD post hoc test used only for specific comparison when indicated by mixed ANOVA, except for the AM251 dose–response curve to which one way ANOVA was applied. Sleep latencies and average duration of REMS episodes were analyzed with one way ANOVA and post hoc LSD. Finally, frequency of REMS was analyzed with Kruskal–Wallis test with post hoc Mann–Whitney *U*-test and Bonferroni correction.

3. Results

3.1. Anandamide and oleamide dose–response curve

Our results showed that the acute administration of 2 μg of anandamide significantly increased REMS in comparison with the CONTROL group and the other doses of anandamide. Statistical analysis by mixed ANOVA indicated a significant group × time interaction for REMS ($F_{7.549,\ 50.32} = 2.489$, $P<0.05$, $\varepsilon = 109$) and differences from the 5th to the 10th hour and from the 18th to the 24th hour with subsidiary ANOVAs (Table 1). The increase in the total time of REMS produced by anandamide was observed from the 5th to the 24th hour, although not all the hours were significant (Fig. 1A). Waking exhibited a reduction in the total time, however non significant (Fig. 1C). Likewise NREM exhibited no significant differences. The time added to the increase of REM sleep was taken from the small non significant reduction of the waking time (Fig. 1B).

On the other hand, acute administration of 25 μg of oleamide significantly increased REMS in comparison to the CONTROL group and the other doses (Fig. 1D to F). Statistical analysis by mixed ANOVA indicated a significant group × time interaction for REMS ($F_{7.75,\ 51.67} = 2.88$, $P<0.05$, $\varepsilon = 0.112$) and differences from the 6th to the 24th hour, although in this case not all the hours were significantly different, with subsidiary ANOVA (Table 2). Similar to anandamide, waking and NREM depicted no significant differences and the time spent on increasing REM sleep was taken from a small fraction of the waking time (Fig. 1D, E, and F).

A group of the dose–response curve of anandamide and oleamide was established at the same time; hence, the CONTROL group is the same for both dose–response curves, therefore the values of this group are plotted in Fig. 1A to F. We decided to illustrate the effects of these eCBs separately for the sake of clarity.

The increase of REMS produced by the administration of 2 μg of anandamide or 25 μg of oleamide was mainly due to an increase in the frequency of REMS episodes rather than to changes in the bouts' mean duration. The acute administration of 2 μg anandamide significantly increased the frequency of bouts in the light phase ($H_3 = 8.49$, $P<0.05$) with respect to the CONTROL group (Fig. 2A). A similar pattern was observed with 25 μg of oleamide, although it did not reach statistical significance.

Table 1
Acute administration anandamide subsidiary ANOVA of REMS.

Hour	Significant *F*
5	$F_{(3,\ 20)} = 3.18^*$
6	$F_{(3,\ 20)} = 3.16^*$
7	$F_{(3,\ 20)} = 3.26^*$
8	$F_{(3,\ 20)} = 3.14^*$
9	$F_{(3,\ 20)} = 3.72^*$
10	$F_{(3,\ 20)} = 3.15^*$
18	$F_{(3,\ 20)} = 3.36^*$
19	$F_{(3,\ 20)} = 3.34^*$
20	$F_{(3,\ 20)} = 4.01^*$
21	$F_{(3,\ 20)} = 4.69^*$
22	$F_{(3,\ 20)} = 5.01^*$
23	$F_{(3,\ 20)} = 5.58^*$
24	$F_{(3,\ 20)} = 5.62^*$

$^*P<0.05$ vs CONTROL.

3.2. Subchronic administration and drug withdrawal

The subchronic administrations (15 days) of anandamide or oleamide were performed by using 2 and 25 μg, respectively. These doses had been effective to increase REMS in their acute administrations.

Our results show that the subchronic administrations of anandamide or oleamide increased REM sleep significantly (Fig. 3A). Mixed ANOVA indicated a significant group × time interaction for REMS ($F_{4.95,\ 37.15} = 3.87$, $P<0.05$, $\varepsilon = 0.108$). Both anandamide and oleamide induced similar effects, although at different doses. It seems that the subchronic administration facilitated even more REMS increase. These eCBs produced a significant increase of REMS from the 5th to 24th hour as shown by subsidiary ANOVA (Table 3). Waking and NREM depicted no significant differences. However, as in the other experiments, the increase of REMS is due to a non significant reduction of the waking time (Fig. 3B and C).

Increased REMS observed after the subchronic administration of anandamide was mainly produced by an increase in the frequency of REMS episodes rather than by changes in the duration of the bouts. Subchronic administration of oleamide significantly increased bouts frequency during the light period ($H_2 = 6.23$, $P<0.05$), and increased the total time ($H_2 = 8.05$, $P<0.05$) (Fig. 4).

On day 16, all rats belonging to any of the three groups were injected with the vehicle. No changes were documented. Rats receiving anandamide or oleamide exhibited a sleep–waking cycle similar to the one of those rats receiving vehicle (Fig. 3D to F).

3.3. AM251 dose–response curve

Fig. 2, B shows the dose–response curve performed to evaluate the effect of AM251 on sleep. The 3.2 μg dose decreased REMS during the first 4 h after its administration with a return to baseline for the 20 following hours. Thus, the data reported here concern only the 4-h period after treatment. Statistical analysis using a one way ANOVA indicates significant differences with the CONTROL group ($F_{3,\ 20} = 3.11$, $P<0.05$).

Although there was a decrease in REMS frequency with the 3.2 μg dose of AM251, significance was not reached. Neither was significant differences observed in the duration of REMS bouts.

3.4. Blockade of acute and subchronic administration of anandamide or oleamide

In an attempt to block anandamide or oleamide effects induced by their acute or subchronic administration on sleep and to further explore the involvement of the CB1 receptor in these effects, we administered 3.2 μg AM251, 15 min before the administration of anandamide or oleamide. As can be seen in Fig. 1G to I and Fig. 3G to I, the administration of the CB1 antagonist receptor blocked the effects of anandamide or oleamide on REMS after their acute or subchronic administration.

Finally, we examined the latencies of NREM and REMS in each of these experiments and found no significant differences regarding the CONTROL groups in any of the latencies.

4. Discussion

Our results indicate that the acute and the subchronic administration of anandamide (2 μg) or oleamide (25 μg) increase REMS. At

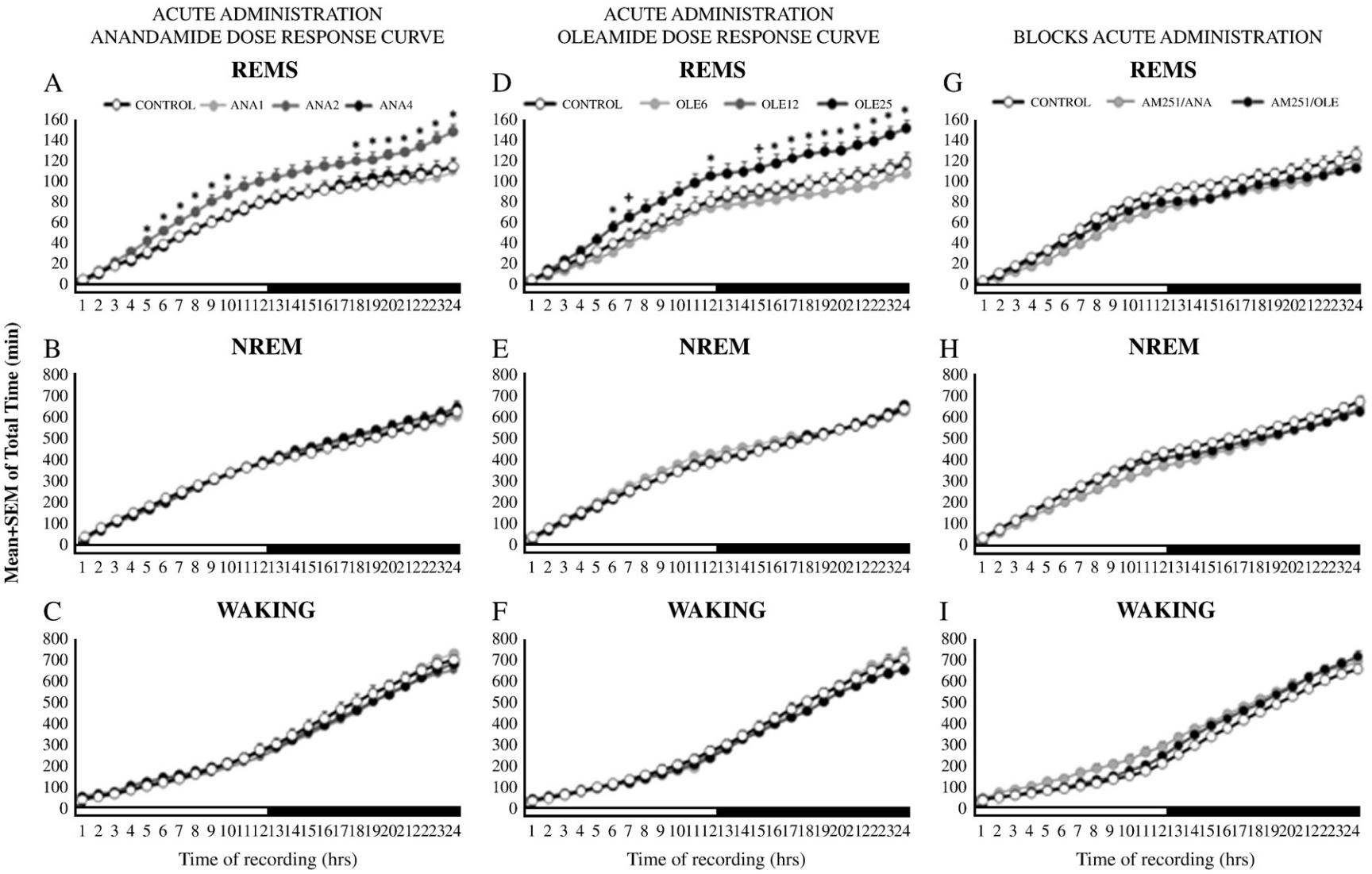

Fig. 1. The left side of this panel illustrates the dose–response curve of the acute administration of 3 doses of anandamide on the sleep–waking cycle, ANA1 (1 μg), ANA2 (2 μg), ANA4 (4 μg), plus the CONTROL group (vehicle). Panel (A) REMS: rapid eye movement sleep. (B) NREM: non-rapid-eye movement sleep. (C) Wakefulness. Panels in the middle of this figure illustrate the dose–response curve of the acute administration of oleamide on the sleep–waking cycle. OLE6 (6 μg), OLE12 (12 μg), OLE25 (25 μg), and CONTROL group (vehicle). (D) REMS: rapid eye movement sleep. (E) NREM: non-rapid-eye movement sleep, and (F) Wakefulness. The CONTROL group is the same for both dose–response curves. Graphs in the right side of this figure illustrate the blockade of the effects induced by the acute administration of anandamide or oleamide on the sleep–waking cycle, by AM251: AM251/ANA (AM251 3.2 μg, 15 min before ANA 2 μg), AM251/OLE (AM251 3.2 μg, 15 min before OLE 25 μg), and CONTROL group (DMSO, 15 min before vehicle saline/ETOH). (G) REMS: rapid eye movement sleep. (H) NREM: non-rapid-eye movement sleep, and (I) Wakefulness. Results are expressed as mean ± SEM of the accumulated time (minutes) of 24 h of recording and the light–dark cycle is indicated by a white–black bar in the *X* axis. Statistical analysis, using a mixed ANOVA test, indicated a significant group × time interaction for REMS, and significant differences for individual time points with a subsidiary ANOVA. Differences against CONTROL group (*$P<0.05$) and "+" differences between OLE6 and OLE12 (+$P<0.05$). Is important to mention some SEM levels are too small to be appreciated in the graphs. No significant differences were detected in the groups receiving AM251.

Table 2
Acute administration oleamide subsidiary ANOVA of REMS.

Hour	Significant *F*
6	$F_{(3,\ 20)}=4.03^{*}$
7	$F_{(3,\ 20)}=3.17^{+}$
12	$F_{(3,\ 20)}=3.12^{*}$
15	$F_{(3,\ 20)}=3.45^{+}$
16	$F_{(3,\ 20)}=4.28^{*}$
17	$F_{(3,\ 20)}=4.86^{*}$
18	$F_{(3,\ 20)}=6.22^{*}$
19	$F_{(3,\ 20)}=7.01^{*}$
20	$F_{(3,\ 20)}=5.96^{*}$
21	$F_{(3,\ 20)}=7.67^{*}$
22	$F_{(3,\ 20)}=7.72^{*}$
23	$F_{(3,\ 20)}=7.78^{*}$
24	$F_{(3,\ 20)}=7.01^{*}$

$^{*}P<0.05$ vs CONTROL.
$^{+}P<0.05$ vs OLE6 and OLE12.

these doses, both eCBs effects are very similar. We have previously shown that the icv administration of anandamide produces REMS increase (Murillo-Rodríguez et al., 2001). In this report we are supporting our previous findings, and in addition, we are reporting for the first time a study describing long-lasting effects, at least for 24 h after a single administration. Moreover, we are showing that eCBs are able to produce a REMS increase for 2 weeks. Oleamide also produced such a long-lasting effect on REMS. Regarding NREM, we did not detect changes induced by these eCBs, despite that we had previously reported that anandamide increases it (Murillo-Rodríguez et al., 2001), and that it has also been reported that oleamide shortens sleep latency (Mendelson and Basile, 1999; 2001), and that its systemic administration increases NREM and total sleep (Laposky et al., 2001). The potential explanation for these discrepancies is that we used different doses of these eCBs in this study as compared with our previous reports. Also, we recorded the EEG from the hippocampus, and this fact might prevent us from observing robust expression of slow waves. In addition, it has come to our attention that the margin to induce changes in the sleep–waking cycle by means of eCBs is very narrow.

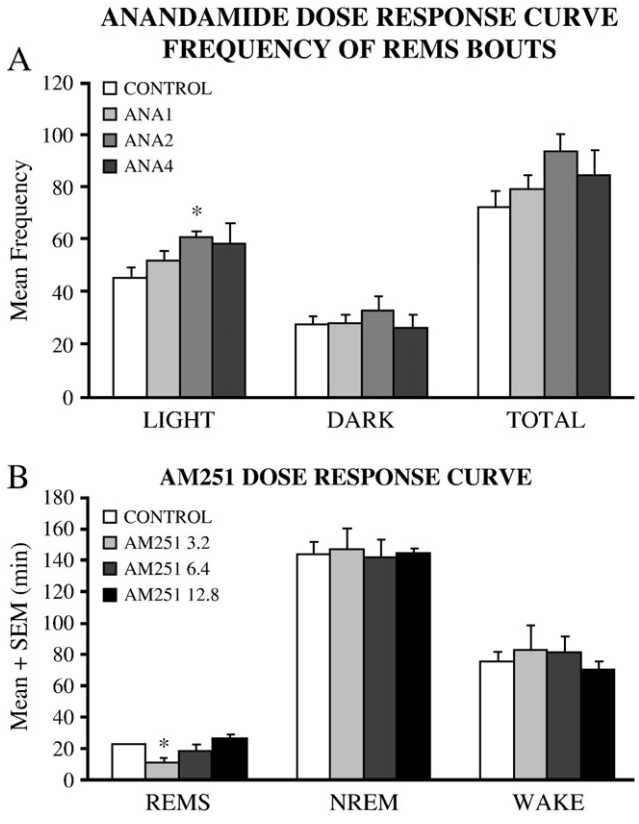

Fig. 2. Panel (A) shows the frequency of REMS bouts in the light and in the dark phase, and during the entire light–dark cycle (Total), under the effect of the different doses used. Results are expressed as mean ± SEM of REMS frequency for 3 doses of anandamide; ANA1 (1 μg), ANA2 (2 μg), ANA4 (4 μg), and CONTROL group (vehicle). Statistical analysis (Kruskal–Wallis test) revealed significant differences between groups, and a Mann–Whitney *U*-test post hoc test indicated significant differences between ANA2 and CONTROL group ($^{*}P<0.05$) in the light phase. Panel (B) shows the dose–response curve of AM251 on the sleep–waking cycle. The results are expressed as mean ± SEM of time (minutes) for the first 4 h of recording for 3 doses of AM251: AM251 3.2 (3.2 μg), AM251 6.4 (6.4 μg), AM251 12.8 (12.8 μg), and CONTROL group (vehicle). A one way ANOVA test indicated significant differences among groups for REMS, and post hoc LSD indicated significant differences between AM251 3.2 and CONTROL groups ($^{*}P<0.05$).

Regarding anandamide's dose–response curve, the highest dose did not produce any effect on sleep. This is not surprising since it has been reported that eCBs induce biphasic effects, like in anxiety, where low doses of anandamide are anxiolytic whereas high doses are anxiogenic (Viveros, et al., 2005). On the other side, administration of AM404 (selective inhibitor of a putative anandamide transporter) produces an inverse U-shaped response in the conditioned place preference test (Bortolato, et al., 2006).

The subchronic administration of anandamide or oleamide increased REMS. Both eCBs caused a similar effect, producing and increase from the 5th hour of administration to the 24th hour, mainly due to an increase in the bouts' frequency. The increase was even higher after the subchronic than after the acute administration. A potential explanation for this phenomenon is that the subchronic administration of anandamide increases the B_{max} of the cannabinoid receptor, as well as the number of receptors (Romero et al., 1995). During acute drug withdrawal, those groups receiving anandamide or oleamide had a sleep–waking cycle similar to the one exhibited by the CONTROL group, indicating that there are no abstinence effects, at least expressed as changes in the sleep–waking cycle. However, it has been reported that oleamide is a poor inducer of physical dependence (Fedorova et al., 2001) and anandamide is capable of producing this effect only at high doses (20 mg/kg, i.p.) (Costa et al., 2000).

Results of the acute and subchronic administration of these eCBs indicate that they may be facilitating somehow REMS triggering mechanisms. However, we have to remark that at the dose presently used (2 μg) we could not reproduce the original effect induced by anandamide at the dose of 1.25 μg (Murillo-Rodríguez et al., 2001).

To support a potential role of the CB1 receptor as mediator of the effects produced by these eCBs on REMS, we used the CB1 receptor antagonist, AM251. Our results showed that AM251 reduces REMS within the first 4 h post administration, supporting the pioneer observations made by Santucci et al. (1996) who administered the CB1 receptor antagonist, SR141716A, causing a decrease of both REMS and NREM, and consequently an increase in waking. Although we recorded 24 h after the administration of AM251, here we are reporting only the first 4 h in which we observed its effect on REM sleep. It is also interesting that AM251 induces the strongest effect with the lowest dose used, and it is less effective with a higher dose. It seems like AM251 causes a pattern of effects similar to CB1 agonists (inverted U shape) but in the opposite direction. We do not have data to explain the mechanisms subserving this pharmacological behavior, but its phenomenological occurrence seems to be warranted.

In this context, blockage of the eCBs effect on REMS lends further support to the role of the CB1 receptor in the generation of REMS, and that eCBs, such as anandamide and oleamide among others not yet tested, are the ones activating this receptor. Our findings also support studies performed by our own group and by others showing that SR141716A blocks the effects produced by anandamide and oleamide on sleep and also prevents REMS rebound (Murillo-Rodríguez et al., 2001; Mendelson and Basile, 1999; Navarro et al., 2003).

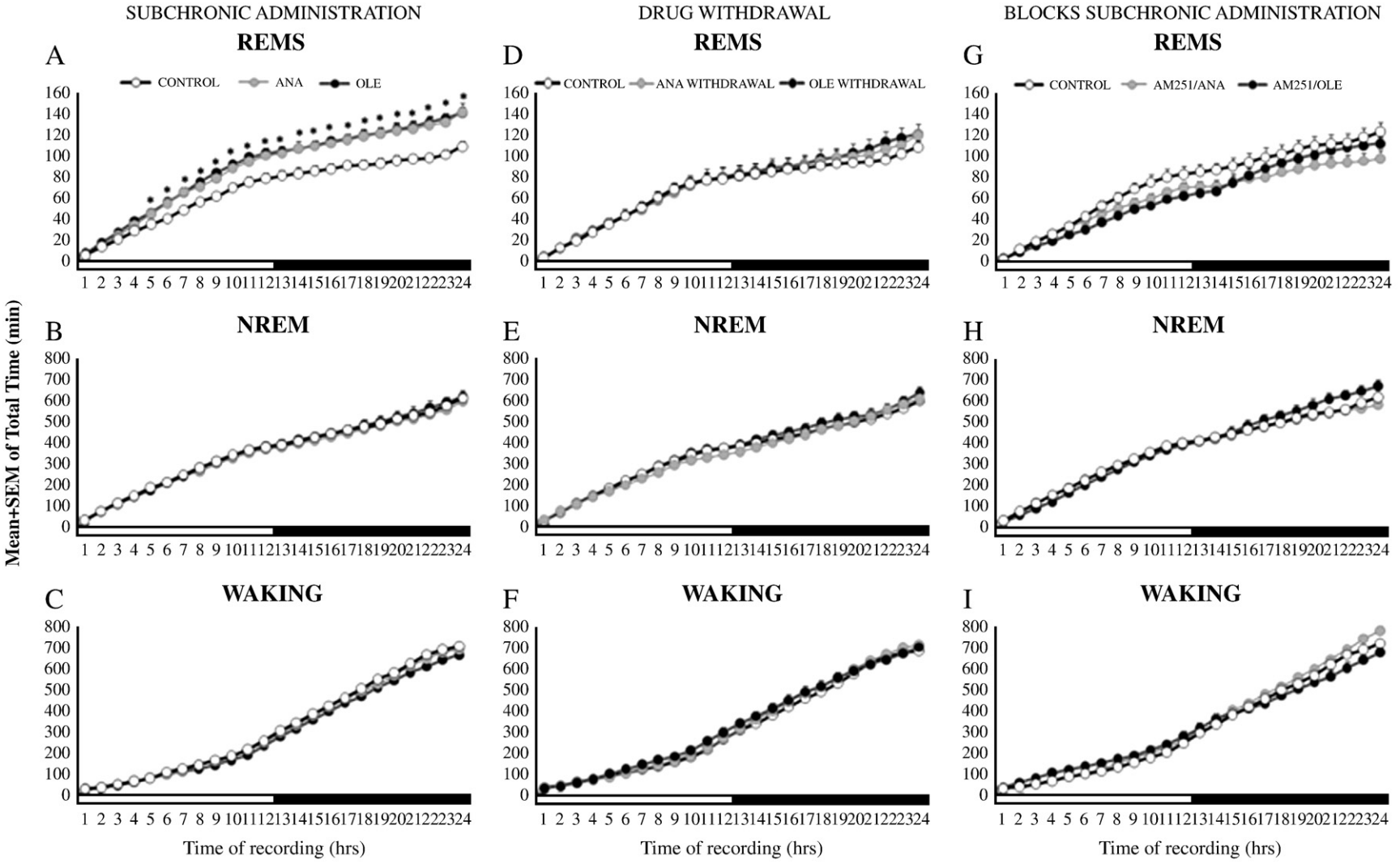

Fig. 3. On the left side of this figure, the effects of the subchronic administration of anandamide or oleamide on the sleep–waking cycle can be observed: ANA (2 μg), OLE (25 μg), and CONTROL group (vehicle). (A) REMS: rapid eye movement sleep. (B) NREM: non-rapid-eye movement sleep, and (C) Wakefulness. Graphs in the middle of this figure illustrate the drugs withdrawal effect on the sleep–waking cycle: ANA WITHDRAWAL, OLE WITHDRAWAL, and CONTROL group (vehicle). These graphs illustrate the effect of substituting anandamide or oleamide by the vehicle, 24 h after the last administration of any of these eCBs. Panel (D) REMS: rapid eye movement sleep (E) NREM: non-rapid-eye movement sleep, and (F) Wakefulness. No statistical differences were found. Graphs on the right side of this figure illustrate the blockade of the effects of the subchronic administration of anandamide or oleamide on the sleep–waking cycle by AM251. Groups were subchronically administered with anandamide, oleamide, or vehicle and, on the 15 day, AM251 was added: AM251/ANA (AM251 3.2 μg, 15 min before ANA 2 μg), AM251/OLE (AM251 3.2 μg, 15 min before OLE 25 μg), and CONTROL group (DMSO, 15 min before vehicle saline/ETOH). (G) REMS: rapid eye movement sleep. (H) NREM: non-rapid-eye movement sleep. (I) Wakefulness. Statistical analysis revealed a lack of significant differences. Results are expressed as mean ± SEM of the accumulated time (minutes) of 24 h of recording and the light–dark cycle is indicated by a white–black bar in the *X* axis. Statistical analysis, using a mixed ANOVA test, indicated a significant group × time interaction for REMS, and significant differences for individual time points with a subsidiary ANOVA. Differences against CONTROL group ($^{*}P<0.05$). It is important to mention some SEM levels are too small to be appreciated in the graphs.

Table 3
Subchronic administration subsidiary ANOVA of REMS.

Hour	Significant *F*
5	$F_{(2, 15)} = 3.88^*$
6	$F_{(2, 15)} = 5.14^*$
7	$F_{(2, 15)} = 6.53^*$
8	$F_{(2, 15)} = 5.86^*$
9	$F_{(2, 15)} = 7.55^*$
10	$F_{(2, 15)} = 7.00^*$
11	$F_{(2, 15)} = 5.49^*$
12	$F_{(2, 15)} = 5.24^*$
13	$F_{(2, 15)} = 4.89^*$
14	$F_{(2, 15)} = 5.67^*$
15	$F_{(2, 15)} = 6.60^*$
16	$F_{(2, 15)} = 7.83^*$
17	$F_{(2, 15)} = 7.55^*$
18	$F_{(2, 15)} = 8.03^*$
19	$F_{(2, 15)} = 8.43^*$
20	$F_{(2, 15)} = 7.47^*$
21	$F_{(2, 15)} = 7.81^*$
22	$F_{(2, 15)} = 7.83^*$
23	$F_{(2, 15)} = 8.38^*$
24	$F_{(2, 15)} = 7.51^*$

$^*P<0.05$ vs CONTROL.

With all, it has been amply shown that eCBs are not the only molecules with sleep-promoting properties. Many proteins are examples of molecules with sleep-inducing properties, i. e. cortistatin, melanin concentrating hormone and many others as we have reviewed elsewhere (Prospéro-García and Méndez-Díaz, 2004). This fact implies that sleep in general depends not only on the action of one family of molecules as eCBs, but on the activity of many more with other chemical nature, i. e. proteins, prostaglandins, and adenosine.

In summary, we have found that both acute and subchronic administrations of anandamide or oleamide increase REMS and that these changes are mainly due to an increase in bouts frequency. In addition, we did not find any signs of acute withdrawal once these eCBs were suddenly suspended, at least not expressed as changes in the sleep–waking cycle. Furthermore, we are providing evidence that these eCBs effects are mediated by the CB1 receptor. In conclusion, this study supports the role of these endogenous cannabinoids in the regulation of REMS.

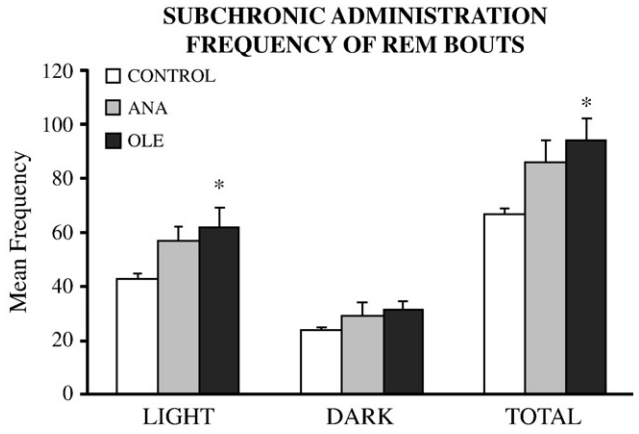

Fig. 4. Illustration of the frequency of REMS bouts in the light phase, dark phase, and during the entire light–dark cycle (Total), for the subchronic administration of anandamide or oleamide. Results are expressed as mean ± SEM of REMS frequency for ANA (2 μg), OLE (25 μg) and CONTROL group (vehicle). Statistical analysis (Kruskal–Wallis test) indicated significant differences between groups and significant differences between groups OLE and CONTROL, in the light phase and the total light–dark cycle ($^*P<0.05$).

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