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Effects of the fatty acid amide hydrolase inhibitor URB597 on the sleep-wake cycle, c-Fos expression and dopamine levels of the rat

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Abstract

Our group has described previously that the endogenous cannabinoid anandamide induces sleep. The hydrolysis of this lipid involves the activity of the fatty acid amide hydrolase (FAAH), which additionally catalyzes the degradation of the satiety factor oleoylethanolamide and the analgesic-inducing lipid palmitoylethanolamide. It has been demonstrated that the inhibition of the FAAH by URB597 increases levels of anandamide, oleoylethanolamide and palmitoylethanolamide in the brain of rats. In order to determinate the physiological properties of the FAAH inhibition on the sleep modulation, we report the pharmacological effects on the sleep–wake cycle of the rat after i.c.v. administrations of URB597, oleoylethanolamide or palmitoylethanolamide (10, 20 μg/5 μl). Separate unilateral i.c.v. injections of 3 compounds during the lights-on period, increased wakefulness and decreased slow wave (SW) sleep in rats in a dose-dependent fashion. We additionally found out that, compared to controls, c-Fos immunoreactivity in hypothalamus and dorsal raphe nucleus was increased in rats that received URB597, oleoylethanolamide or palmitoylethanolamide (10, 20 μg/5 μl, i.c.v.). Next, we found that after an injection of the compounds, levels of dopamine were increased whereas extracellular levels of levodopa (L-DOPA) were decreased. These findings indicate that that inhibition of the FAAH, via URB597, modulates waking. These effects were mimicked separately by the administration of oleoylethanolamide or palmitoylethanolamide. The alertness induced by the compounds tested here activated wake-promoting brain regions and they also induced the release of dopamine. Our results suggest that FAAH activity as well as two molecules that are catalyzed by this enzyme, oleoylethanolamide and palmitoylethanolamide, participate in the regulation of the waking state. Alternative approaches to treat sleep disorders such as excessive somnolence might consider the use of the URB597, oleoylethanolamide or palmitoylethanolamide since all compounds enhance waking.

Keywords: Wakefulness; Anandamide; Microdialysis; Rapid eye movement sleep; Oleoylethanolamide; Hypothalamus

1. Introduction

The National Highway Traffic Safety Administration in The United States of America estimates that nearly every year 100,000 police-reported crashes are related to drowsy drivers cases. According to the National Sleep Foundation poll, 60% of Americans said they drove while feeling drowsy during 2005. This alarming data highlights the need to identify the molecular and cellular mechanisms involved in control of drowsiness.

One such mechanism, may involve the family of lipid-signalling compounds that includes arachidonoylethanolamine (anandamide), the first endogenous agonist for cannabinoid receptors identified (Devane et al., 1992), oleoylethanolamide (Rodriguez de Fonseca et al., 2001) and palmitoylethanolamide (Calignano et al., 2001).

It is known that pharmacologically, anandamide mimics many of the effects caused by Δ^9-tetrahydrocannabinol, the primary psychoactive molecule in marijuana (Gaoni and Mechoulam, 1964), on diverse behaviours such as memory disruption, hypolocomotion hyperphagia, and modulates sleep (Fride and Mechoulam, 1993; Smith et al., 1994; Wiley et al.,

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1995; Murillo-Rodríguez et al., 1998, 2001, 2003; Williams and Kirkham, 1999).

Among the evidence of the biological properties of oleoylethanolamide, our group has described recently its diurnal variation in cerebrospinal fluid as well as in different brain regions (Murillo-Rodríguez et al., 2006a). On the other hand, this lipid has little or no effect on formalin-evoked pain behavior (Calignano et al., 2001) and it has been proposed that it might be involved in mechanisms of satiety (Inui, 2004). Its anorexic action in rats is not blocked by cannabinoid receptors-specific antagonists (Rodriguez de Fonseca et al., 2001). It has described that peripheral administrations of oleoylethanolamide delayed feeding onset in a dose-dependent manner (Gaetani et al., 2003). This anorexic effect involves a peroxisome proliferator-activated receptor-alpha (PPAR-α; Fu et al., 2003).

Recent studies report that palmitoylethanolamide displays some biological properties (Lambert et al., 2002). For instance, Calignano and co-workers (2001) showed that this compound acted as an antinociceptive molecule. Capasso et al. (2001) have reported that palmitoylethanolamide significantly decrease intestinal transit, and its anti-inflammatory properties have been described as well (Costa et al., 2002; Lo Verme et al., 2005b).

The hydrolysis of anandamide, oleoylethanolamide and palmitoylethanolamide is catalyzed by an intracellular enzyme named the fatty acid amide hydrolase (FAAH, McKinney and Cravatt, 2005). The inhibition of the FAAH by highly selective inhibitors (Tarzia et al., 2003; Mor et al., 2004) such as cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester (URB597) increases the endogenous levels of anandamide, oleoylethanolamide and palmitoylethanolamide in the rat brain (Fegley et al., 2005).

Since we have reported that injections of anandamide increase sleep (Murillo-Rodríguez et al., 1998, 2001, 2003), we hypothesized that the inhibition of the FAAH via URB597 would increase the endogenous levels of anandamide, as reported by Fegley et al. (2005), and it would therefore lead to an enhancement in sleep. In order to test this hypothesis, we injected i.c.v. URB597 (10, 20 μg/5 μl) and sleep recordings were analyzed. Additionally, we also injected i.c.v. oleoylethanolamide (10, 20 μg/5 μl) or palmitoylethanolamide (10, 20 μg/5 μl) to compare the effects on sleep among the drugs. The molecular pathways that possibly activate these drugs were analyzed via c-Fos immunoreactivity. In addition, since URB597 increases intracellular levels of anandamide as mentioned above, it might be possible that dopaminergic system could be involved in the effects caused by URB597 on sleep. It has been pointed out that anandamide significantly increase dopamine extracellular levels (Hao et al., 2000). Therefore, in the present study, we also measured the extracellular levels of dopamine collected from nucleus accumbens using microdialysis and measured by HPLC after URB597, oleoylethanolamide or palmitoylethanolamide injection.

2. Materials and methods

2.1. Animals

Male wistar rats ($n=130$; 250–300 g) were housed at constant temperature (21±1 °C) and under a controlled light–dark cycle (lights on: 07:00–19:00 h). Food and water were provided *ad libitum*. All experimental procedures were carried out according to a protocol approved by the local Animal Ethics Committee.

2.2. Surgery, EEG/EMG electrodes and i.c.v. cannulae

Animals ($n=80$) were implanted under deep anesthesia (acepromazine [0.75 mg/kg], xylazine [2.5 mg/kg], and ketamine [22 mg/kg, i.p.] for sleep recordings and a cannula (23gauge) was placed into one lateral ventricle ($A=-0.8$; $L=-1.6$; $H=-3.6$; Paxinos and Watson, 1986). All electrodes and cannulae were placed and secured onto the skull using dental cement. The procedures have been reported previously by our group (Murillo-Rodríguez et al., 1998, 2001, 2006b). After the surgeries, all animals were placed into the sleep-recording chambers for habituation with food and water *ad libitum*.

2.3. Surgery, microdialysis guide-cannulae and i.c.v. cannulae

A different group of rats was used for the microdialysis study ($n=50$). A guide-cannula (IC guide. BioAnalytical Systems [BAS], West Lafayette, IN, USA) was placed stereotaxically into the Accumbens nucleus, core (AcbC; target coordinates: $A=+1.2$; $L=2.0$; $H=-7.0$; [Paxinos and Watson, 1986]). Additionally, a cannula (23 gauge) was placed into one lateral ventricle as described above. The guide-cannulae and i.c.v. cannulae were then fixed onto the skull with a thin layer of dental cement. After surgery, each animal was placed into the Microdialysis Bowl to habituate them to the experimental conditions. All animals were allowed to recover for at least 7 days after all surgeries. All experiments were conducted with food and water available *ad libitum*.

2.4. Pharmacological administrations

URB597, oleoylethanolamide and palmitoylethanolamide were kindly provided by Professor Daniele Piomelli (University of California, Irvine. USA). The compounds were dissolved in a vehicle (PEG/saline; 5:95 v/v). In order to test the pharmacological properties of the compounds on sleep during the lights-on or lights-off period, they were injected i.c.v. as follows: Vehicle (control group), URB597 (10, 20 μg/5 μl), oleoylethanolamide (10, 20 μg/5 μl) or palmitoylethanolamide (10, 20 μg/5 μl) at 07:00 h or at 19:00 h.

The doses were injected randomly to the rats and for the following injections, a period of 3 days was chosen between administrations. All pharmacological administrations were done slowly over 1 μl/min with the injector left in the target for an additional 15 s to ensure extrusion from the tip and to minimize distribution of treatments upwards on the cannulae. After all injections, the cannula was withdrawn and the stylet replaced. Right after microinjections, animals were attached to either the sleep-recording or the microdialysis system. In the sleep-wake experiments, an additional group was added (sham group) in order to determinate if the vehicle injection per se could modify the sleep-wake cycle of the rats.

2.5. Analyses of sleep recordings

The EEG/EMG data recordings were scored manually in epochs for wakefulness, slow wave (SW) sleep and rapid eye movement (REM) sleep as described previously (Murillo-Rodríguez et al., 1998, 2001). The analysis was restricted to 4h after injections since we know pharmacokinetics of URB597 are rapidly absorbed. The effect of URB597 on FAAH activity is within 2 h post-systemic administrations according to Fegley et al. (2005).

2.6. Microdialysis sampling procedures

One week after surgery and a day before the experiment, the microdialysis probe (1 mm of length. Polyacrylonitrile, MWCO=30,000 Daltons; 340 μm OD; BAS) was inserted through the guide cannula into the target structure at 7:00 h and the tissue was allowed to stabilize for 24 h. During this period artificial cerebrospinal fluid (aCSF, composition: NaCl (147 mM), KCl (3 mM), CaCl (1.2 mM), MgCl (1.0 mM), pH 7.2)) was perfused through a FEP Teflon Tubing (0.65 mm OD×0.12 mm ID) continuously using a 2.5 mL gastight syringe. All procedure reported previously in Murillo-Rodríguez et al. (2003). The syringe Pump (CMA/100) controlled the ACSF perfusion (flow rate: 1 μl/min). We included a sham group ($n=10$) in order to determinate if the injection of the vehicle (control; $n=10$) could modify the contents of dopamine. Since we found behavioral changes in sleep after URB597, oleoylethanolamide or palmitoylethanolamide injection during the lights-on period, we exclusively collected dialysates every hour during the lights-on phase.

2.7. Analysis of dopamine

Immediately after collection samples were injected into a HPLC (Gilson) for dopamine analysis. Briefly, the mobile phase consisted of monochloacetic acid (0.1 M), sodium octylsulfate (223 mM) and disodium ethylenediaminetetracetate dehydrate (0.5 mM) with a flow rate of 80 μl/min. Separation was achieved by cation exchange using a BAS microbore precolumn and column (biophase octyl, 5 μm, 10×1 mm microbore; BAS). Electrochemical detection was performed via BAS LC 3C detector. Chromatographic data was recorded in a PC computer and peak heights of dopamine in microdialysis samples were compared to standards. Dopamine peaks were initially identified by running samples (also 20 μl) that contained different concentrations of dopamine. All details of procedure have been reported by our group recently (Murillo-Rodríguez et al., 2006b).

2.8. Immunohistochemical study

At the end of the experiments, animals that were used in the microdialysis experiment were given a lethal dose of pentobarbital and sacrificed 60 min after receiving an i.c.v. injection at 07:00 h of either the vehicle ($n=5$), URB597 ($n=5$; 10 μg/5 μl), oleoylethanolamide ($n=5$, 10 μg/5 μl) or palmitoylethanolamide ($n=5$, 10 μg/5 μl). They were perfused transcardially with 0.9% saline solution followed by paraformaldehyde followed by 20% sucrose-0.1 M PBS for 48 h. The brains were cut (frozen sections, 30 μm, coronal) and collected in 1:5 serial orders. Immunohistochemistry for c-Fos was done as described in detail by our group (Murillo-Rodríguez et al., 2006b). Additionally to the c-Fos study, sections were lightly counterstained with Neutral Red to allow better visualization of the cannula track for the microdialysis experiments. We did not include a sham group since no statistical differences were found in the behavioural and biochemical studies among these groups. All studies were conducted in accordance with the principles and procedures described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.9. Statistical analysis

The data are presented as mean±SEM. Statistical significance was evaluated using one-way analysis of variance (ANOVA), followed by the Sheffé test as a post-hoc test (STATVIEW, $P<0.05$).

3. Results

3.1. Effects of URB597, oleoylethanolamide or palmitoylethanolamide on sleep

In experiment 1, we found no statistical differences on the sleep-wake cycle among the sham ($n=8$) and control ($n=8$) group. Next, we injected either URB597 ($n=8$; 10, 20 μg/5 μl) or vehicle ($n=8$) during the rat's normal sleeping period (07:00 h, lights-on period). URB597 at the doses tested markedly increased dose-dependently the total time spent in wakefulness ($DF=28$; $F=6.660$; $P<0.01$; Fig. 1A). We also found that this drug induced a dose-response diminution in SW sleep ($DF=28$; $F=6.618$; $P<0.02$; Fig. 1A) whereas REM sleep remained with no statistical change. URB597 (10 μg/5 μl) injected at the beginning of the lights-off period did not modify the total time of wakefulness, SW sleep and REM sleep (data not shown).

The hourly pharmacological effects of URB597 (10, 20 μg/5 μl) on sleep are shown in Fig. 1B, C and D. We found that this drug enhanced wakefulness and the effect was seen on the first ($DF=28$; $F=4.243$; $P<0.04$; Fig. 1B) and on the second hour post-injection ($DF=28$; $F=4.281$; $P<0.001$; Fig. 1B). The doses tested induced effects on SW sleep hourly that are displayed in Fig. 1C. After injection of the drug, changes were observed in the first ($DF=28$; $F=4.489$; $P<0.02$) and the second hour post-injection ($DF=28$; $F=3.551$; $P<0.02$). REM sleep displayed a decrease only in the second hour post-administration of the drug ($DF=28$; $F=3.459$; $P<0.02$; Fig. 1D).

Since it was shown that URB597 indeed increases the endogenous levels of anandamide but the rates are higher for oleoylethanolamide and for palmitoylethanolamide (Fegley et al., 2005), we next investigated whether the increase in wakefulness after URB597 injection could be due to an endogenous accumulation of oleoylethanolamide and/or palmitoylethanolamide. During the lights-on period, the i.c.v administration of oleoylethanolamide ($n=6$; 10, 20 μg/5 μl) compared to the sham

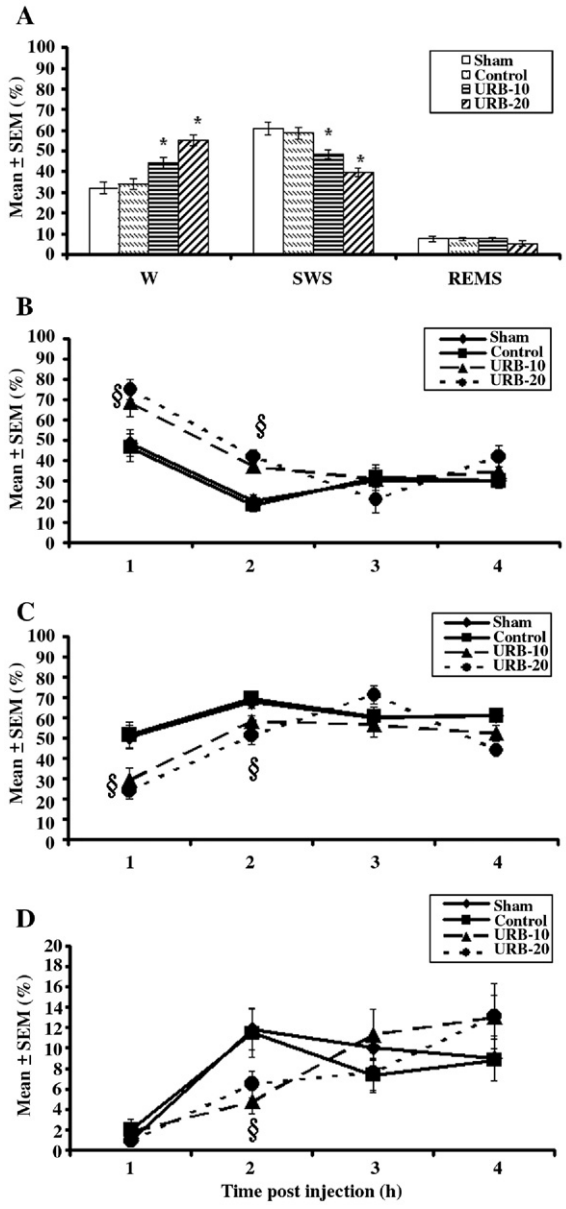

Fig. 1. Effects of total time (4h of sleep recordings) on wakefulness (W), slow wave sleep (SWS) and rapid eye movement sleep (REMS) after i.c.v. administrations of either vehicle (control) or URB597 (10,20 μg/5 μl) during the lights-on (A). Each point represents the mean±SEM of total time of recording (%; * vs. Control, $P<0.05$). Panel B shows the hourly effects on waking of URB597 (10, 20 μg/5 μl) during the lights-on period whereas panels C and D display the effects hour by hour on SWS and REMS, respectively. Each point represents the mean±SEM of total time of recording (%; § (URB-10, 20 μg/5 μl) vs. Control, $P<0.05$; * vs. Control, $P<0.05$).

($n=6$) and control group ($n=6$) mimicked the effects caused by URB597 on the sleep–wake cycle. As shown in Fig. 2A, we found a significant dose-dependent increase in wakefulness ($DF=20$; $F=2.746$; $P<0.02$) whereas SW sleep presented a diminution ($DF=20$; $F=3.007$; $P<0.01$) and REM sleep showed a significant decrease ($DF=20$; $F=2.855$; $P<0.01$). Those effects were analyzed hourly, and we found that oleoylethanolamide (10, 20 μg/5 μl) increased wakefulness in a dose-dependent fashion during the first ($DF=20$; $F=3.820$; $P<0.005$; Fig. 2B) and the last hour post-injection ($DF=20$; $F=3.044$; $P<0.01$; Fig. 2B). A decrease in wakefulness was found in the second hour post-injection with both doses tested ($DF=20$; $F=3.555$; $P<0.05$). Fig. 2C shows that in the doses tested, SW sleep was diminished by the first hour ($DF=20$; $F=3.879$; $P<0.004$) whereas using the highest dose, the last hour showed a significant decrease ($DF=20$; $F=3.027$; $P<0.01$). Unexpectedly, both doses enhanced SW sleep at the second hour post-administration ($DF=20$; $F=2.212$; $P<0.001$). Finally, we found that the highest dose of

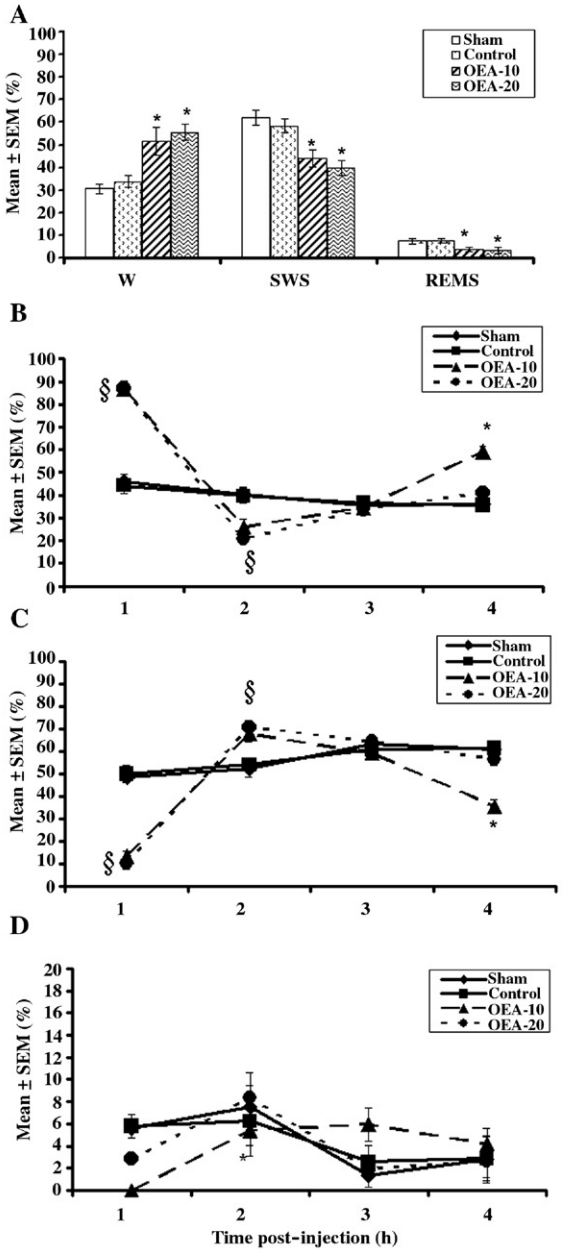

Fig. 2. Effects on total time (4 h of sleep recordings) after i.c.v. administrations of either vehicle (control) or oleoylethanolamide (OEA;10, 20 μg/5 μl) during the lights-on period on wakefulness (W), slow wave sleep (SWS) and rapid eye movement sleep (REMS; A). The hourly pharmacological effects of oleoylethanolamide on W, SWS and REMS are shown in panels B, C and D, respectively. Each point represents the mean±SEM of total time of recording (%; § (OEA-10, 20 μg/5 μl) vs. Control, $P<0.05$; * vs. Control, $P<0.05$).

oleoylethanolamide decreased REM sleep in the second hour post-injection ($DF=20$; $F=3.001$; $P<0.01$; Fig. 2D). No statistical changes on sleep stages were observed after the i.c.v. injection of oleoylethanolamide (10 μg/5 μl) during the lights-off period (data not shown).

During the lights-on period, a different group of rats received vehicle (control, $n=6$) or palmitoylethanolamide ($n=6$; 10, 20 μg/5 μl). Compared to the sham ($n=6$) or control group, palmitoylethanolamide induced a similar effect as obtained after URB597 or oleoylethanolamide injection. As shown in Fig. 3A, PEA (both doses) enhanced wakefulness ($DF=20$; $F=2.796$; $P<0.02$) whereas SW sleep ($DF=20$; $F=2.182$; $P<0.01$) as well as REM sleep ($DF=20$; $F=3.339$; $P<0.01$) were found diminished. The hourly analysis of wakefulness showed that palmitoylethanolamide increased it in a dose-dependent fashion during the first hour post-injection ($DF=20$; $F=2.800$; $P<0.05$; Fig. 3B). A significant diminution in SW sleep (Fig. 3C) was observed in the first hour post-injection of both doses of palmitoylethanolamide ($DF=20$; $F=2.225$; $P<0.01$) whereas the highest dose increased REM sleep in the second hour post-injection ($DF=20$; $F=3.258$; $P<0.01$; Fig. 3D). No statistical changes on sleep were observed after palmitoylethanolamide injection (10 μg/5 μl) during the lights-off period (data not shown).

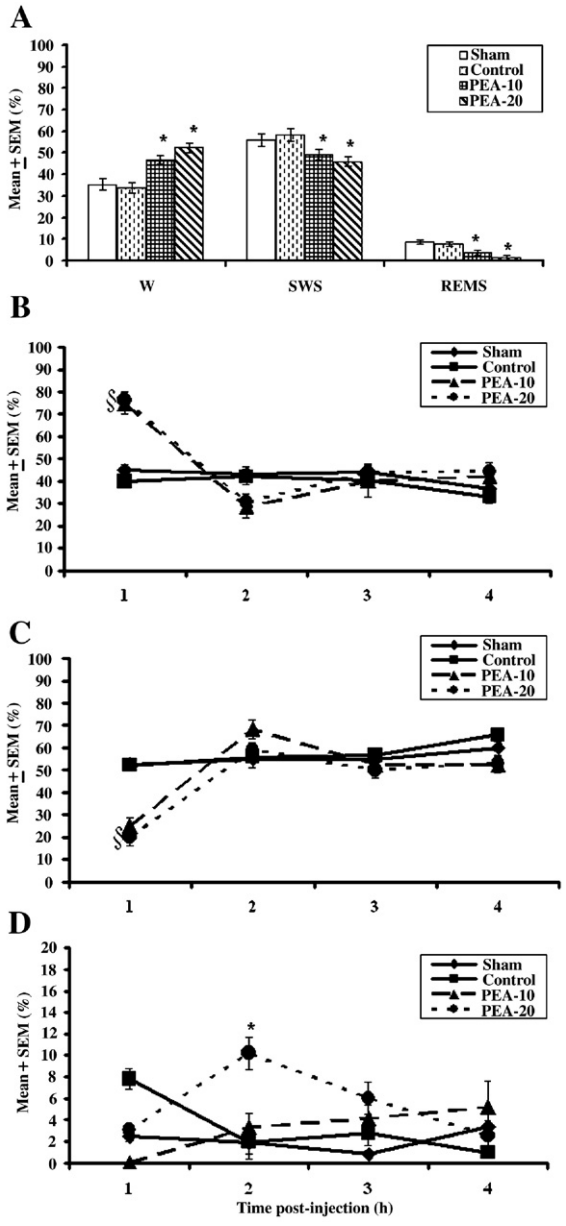

Fig. 3. Effects on total time (4h of sleep recordings) after i.c.v. administrations of either vehicle (control) or palmitoylethanolamide (PEA; 10, 20 μg/5 μl) during the lights-on period on wakefulness (W), slow wave sleep (SWS) and rapid eye movement sleep (REMS; A). The hourly pharmacological effects of palmitoylethanolamide on W, SWS and REMS are shown in panels B, C and D, respectively. Each point represents the mean ± SEM of total time of recording (%; § (PEA-10, 20 μg/5 μl) vs. Control, $P<0.05$; * vs. Control, $P<0.05$).

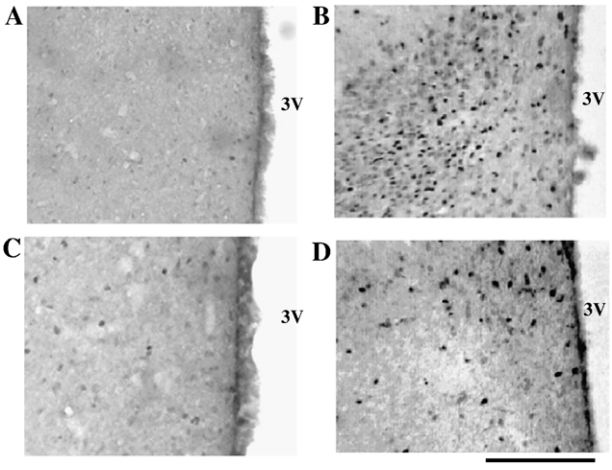

Fig. 4. Localization of c-Fos expression in the rat hypothalamus. Immunohistochemical staining was performed in 30 μm brain section cut of the coronal plane. A, density of c-Fos expression from a control animal; B, staining obtained from experimental animal (URB597, 10 μg/5 μl, i.c.v.); C, c-Fos expression from a rat that received oleoylethanolamide (10 μg/5 μl, i.c.v.) and D, staining from a palmitoylethanolamide-treated rat (10 μg/5 μl, i.c.v.). Note the increase in c-Fos expression in hypothalamus from the animal that received URB597, oleoylethanolamide and palmitoylethanolamide compared to control. Abbreviations: 3V, 3rd ventricle. Scale bar, 100 μm.

3.2. c-Fos expression in the hypothalamus after URB597, oleoylethanolamide or palmitoylethanolamide injection

Compared to their respective control, the treatment with URB597 ($n=5$; 10 μg/5 μl, i.c.v.), oleoylethanolamide ($n=5$; 10 μg/5 μl, i.c.v.) or palmitoylethanolamide ($n=5$; 10 μg/5 μl, i.c.v.) consistently increased the pattern of neuronal activation marked by Fos expression in some brain regions, such as the lateral hypothalamus and dorsal raphe nucleus.

Some of the most striking changes in c-Fos expression were found within specific hypothalamic areas implicated in the alertness control (Fig. 4). Neurons of the hypothalamus are active during wakefulness (Szymusiak et al., 1998), and because all animals were mainly awake after the injections of the compounds, it was not surprising that c-Fos-immunoreactivity hypothalamic neurons were activated in rats that received URB597, oleoylethanolamide or palmitoylethanolamide. In hypothalamic areas, the compounds produced a moderate increase in c-Fos expression within the lateral hypothalamic area

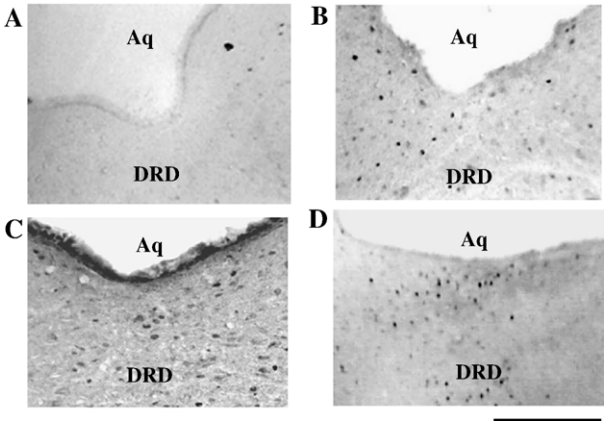

Fig. 5. Expression of c-Fos in dorsal raphe in the rat. Brains were cut at 30 μm of the coronal plane. A, control rat; B, experimental rat (URB597, 10 μg/5 μl, i.c.v.); C, oleoylethanolamide-treated rat (10 μg/5 μl, i.c.v.), and D, c-Fos expression from a animal that received palmitoylethanolamide (10 μg/5 μl, i.c.v.). Abbreviations: Aq: aqueduct (Sylvius), DRD: dorsal raphe nucleus, dorsal part. Scale bar, 100 μm.

and the lateroanterior nucleus. URB597-, oleoylethanolamide- or palmitoylethanolamide-treated rats had more Fos immunoreactivity in the lateral part of the hypothalamus than did the control group (Fig. 4B, C and D, respectively).

3.3. c-Fos expression in the dorsal raphe nucleus after URB597, oleoylethanolamide or palmitoylethanolamide injection

On the other hand, in the brainstem, the dorsal raphe nucleus contained only Fos-immunoreactive neurons with significant difference after treatment with URB597 (10 μg/5 μl, i.c.v.), oleoylethanolamide (10 μg/5 μl, i.c.v.) or palmitoylethanolamide (10 μg/5 μl, i.c.v.) compared with the respective control as shown in Fig. 5 (Panels B, C and D, respectively). No differences were evident in other brainstem nuclei including the pedunculopontine, laterodorsal tegmental nuclei and locus coeruleus.

3.4. Effects of URB597, oleoylethanolamide or palmitoylethanolamide on dopamine levels

Fig. 6A represents a drawing taken from the atlas (Paxinos and Watson, 1986) that identifies the location of the track of the microdialysis probe in the AcbC nucleus as represented by a black bar. Microdialysates were collected and dopamine was measured from AcbC (as the track probe shows; Fig. 6B). In our surgeries, the variation in location of the cannula across the rats was as follows: anterior–posterior was Bregma +1.70 mm to Bregma 0.70 mm; lateral was 1.5 mm to 2.0 mm and dorsal–ventral was 7.5 mm to 7.9 mm. In all animals, the microdialysis probe was placed within the target area.

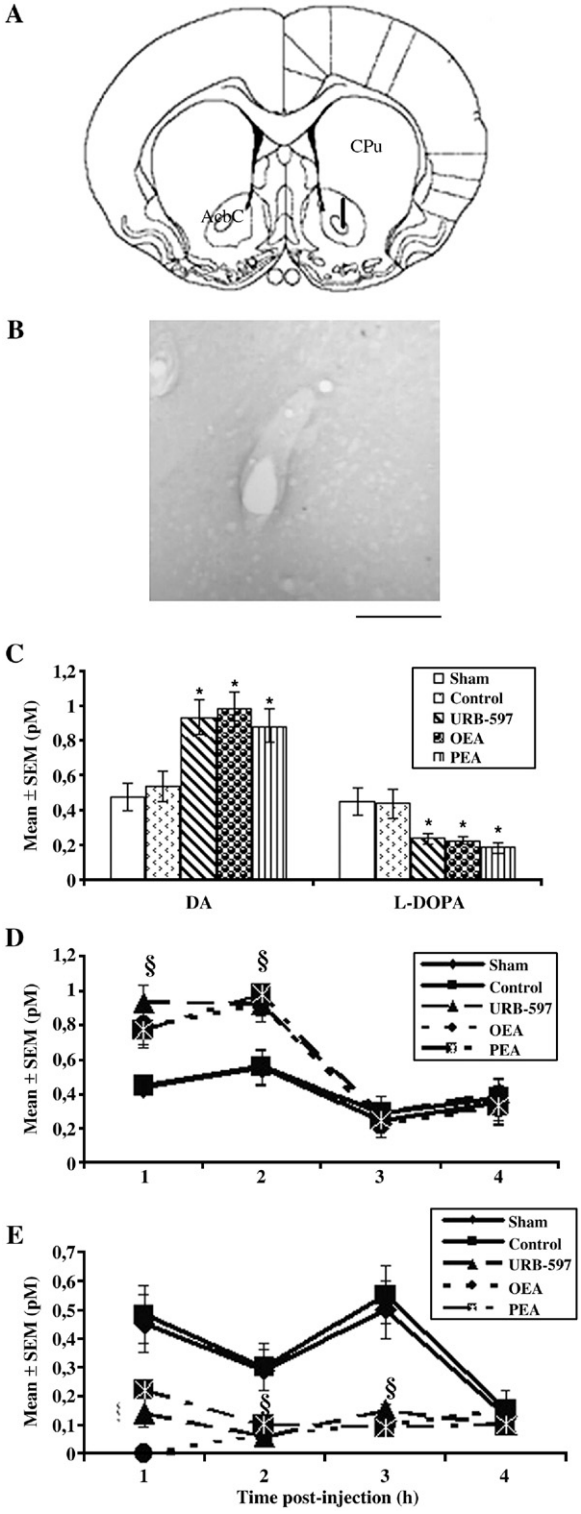

Fig. 6. Schematic representation of the localization of the microdialysis probe in the nucleus accumbens (AcbC). Stereotaxic coordinates, drawings and abbreviations were taken from the Paxinos and Watson (1986) atlas (A). Photomicrograph of the track of the microdialysis probe in the AcbC (B). Extracellular levels of dopamine (DA) and L-DOPA were collected from the AcbC during the lights-on period after i.c.v. injection of either vehicle (control), URB597 (10 μg/5 μl), oleoylethanolamide (10 μg/5 μl), or palmitoylethanolamide (10 μg/5 μl). Levels of DA and L-DOPA (C) after the injections of compounds were found increased and decreased, respectively whereas the hourly analysis of DA and L-DOPA after the treatments is shown in panels D and E, respectively. Abbreviations: AcbC: nucleus accumbens, core; CPu, caudate putamen. Each point represents the mean±SEM of pM (§ (URB-, OEA-and PEA-10, μg/5 μl) vs. Control, $P<0.05$; * vs. Control, $P<0.05$). Scale bar, 100 μm.

We performed the microdialysis experiments in order to determine the functional properties of URB597 (10 μg/5 μl, i.c.v.), oleoylethanolamide (10 μg/5 μl, i.c.v.) or palmitoylethanolamide (10 μg/5 μl, i.c.v.) on the extracellular levels of dopamine. Fig. 6C shows that the levels of dopamine were enhanced after the injection of URB597 ($n=10$), oleoylethanolamide ($n=10$) or palmitoylethanolamide ($n=10$; $DF=45$; $F=2.453$; $P<0.004$) whereas the contents of L-DOPA were diminished after the injection of the 3 compounds ($DF=45$; $F=3.945$; $P<0.02$; Fig. 6C).

Additionally, the hourly analysis showed that separately, the 3 compounds also induced a significant enhancement in dopamine concentration 1 h post-injection ($DF=45$; $F=4.221$; $P<0.003$; Fig. 6D). This effect was observed during the second hour as well ($DF=45$; $F=3.700$; $P<0.002$; Fig. 6D). On the other hand, L-DOPA extracellular levels were decreased 1 h ($DF=45$; $F=6.789$; $P<0.01$; Fig. 6E) as well as in the second ($DF=45$; $F=3.666$; $P<0.03$; Fig. 6E) and in the third hour ($DF=45$; $F=8.312$; $P<0.05$; Fig. 6E) post-injection of URB597, oleoylethanolamide or palmitoylethanolamide.

4. Discussion

The fatty acid amide hydrolase (FAAH) is an intracellular enzyme responsible for the hydrolysis of molecules such as the endocannabinoid anandamide as well as the satiety lipid oleoylethanolamide and the analgesic-inducing factor palmitoylethanolamide (McKinney and Cravatt, 2005). Even though the molecules' effects on sleep, catalyzed by FAAH, such as anandamide, have been reported (Murillo-Rodríguez et al., 1998, 2001, 2003), no evidence was available about the physiological properties of URB597, oleoylethanolamide or palmitoylethanolamide on sleep modulation. The present experiment gives an analysis of the pharmacological effect of the compounds mentioned above on the sleep of rats.

In our conditions, i.c.v. administrations of URB597 during the lights-on period increased wakefulness in a dose-dependent fashion (Fig. 1A). The pharmacological properties of URB597 in the sleep–wake cycle were observed within the 2 h post-injection (Fig. 1B, C and D). These effects on sleep were mimicked by oleoylethanolamide at two different doses (10, 20 μg/5 μl, see Fig. 2A, B, C and D). We additionally found that palmitoylethanolamide (10, 20 μg/5 μl) also increased wakefulness and reduced SW sleep and REM sleep (Fig. 3A).

Importantly, we found out that the hourly analysis showed that the time frame needed for the effects observed in waking after injection of URB597, oleoylethanolamide or palmitoylethanolamide was about 1 h. This data may suggest that the pharmacological effects are within a time window of 60 min after administrations; due possibly to a fast degradation of the compounds tested in the present study.

Irrespective of possible explanations, our findings clearly indicate that URB597 increased waking (Fig. 1). An unexpected result indeed, one might think that URB597 would increase sleep. Our results suggest that the effects on sleep caused by this drug might be due to the enhancement in the intracellular levels of oleoylethanolamide and/or palmitoylethanolamide.

The discovery of oleoylethanolamide as an agonist with high-affinity to the PPAR-α (Fu et al., 2005; Lo Verme et al., 2005a) raises the hypothesis that this could be the molecular mechanism of oleoylethanolamide to modulate waking state. In central nervous system, PPAR-α has been localized in restricted brain areas (Kainu et al., 1994; Cimini et al., 2005). The role of PPAR-α has mainly been related to lipid metabolism but these receptors have been implicated in neural cell differentiation, inflammation and neurodegeneration (Kitamura et al., 1999; Saluja et al., 2001) and related to feeding (Lo Verme et al., 2005a). The possible mechanisms include the transient receptor potential vanilloid type 1 (TRPV1; Ahern, 2003) and recently reported the Ras/extracellular signal regulated kinase (Erk) signalling, at least in part through activation of Neu/ErbB2, and rendered a cardiac protective effect (Su et al., 2006). Despite the evidence that oleoylethanolamide activates the systems mentioned above, the mechanism of action of oleoylethanolamide modulating the sleep–wake cycle remains unclear.

On the other hand, palmitoylethanolamide is synthesized during inflammation and tissue damage (Re et al., 2005). The mechanism of action of palmitoylethanolamide remains to be elicited; however, a hypothesis has been raised that this lipid activates the cannabinoid receptor system. The cannabinoid CB_1 receptor, first identified by Devane et al. (1988), and later cloned by Matsuda et al. (1990), is expressed in different brain areas and in peripheral tissues (Munro et al., 1993). Later, a different protein that recognizes the cannabinoids was identified and named cannabinoid CB_2 receptor. This new receptor has been classified as "peripheral" since its mRNA was detected in inflammatory and immune system cells. The hypothesis that palmitoylethanolamide could be acting on a "cannabinoid CB_2-like receptor" suggests that pharmacological effects of palmitoylethanolamide may be the result of direct or indirect stimulation of cannabinoid CB_2 receptors (or of a yet uncharacterised cannabinoid CB_2-like receptor). In support of this idea, palmitoylethanolamide was initially reported to displace the binding of the high-affinity cannabinoid agonist [^{3}H]WIN55,212-2 with a half-maximal inhibitory concentration (IC_{50}) of 1.0 nM from RBL-2H3 cell membranes, which are known to express cannabinoid CB_2 mRNA (Facci et al., 1995). However, these results have not been subsequently replicated by different groups (Jacobsson and Fowler, 2001; Lambert et al., 2002; Lambert and Di Marzo, 1999; Showalter et al., 1996; Sugiura et al., 2000). Therefore, at the present date it is accepted that palmitoylethanolamide does not bind to the cannabinoid CB_2 receptor. Despite this evidence, it has been reported that the administration of SR144528, a cannabinoid CB_2 specific receptor antagonist, eliminates the antinociceptive effects of palmitoylethanolamide (Calignano et al., 1998, 2001; Conti et al., 2002; Farquhar-Smith and Rice, 2003).

An alternative hypothesis leads to palmitoylethanolamide possibly acting on a different receptor. For instance, as mentioned previously, oleoylethanolamide regulates feeding behavior and the lipid metabolism in rodents by activating the PPAR-α (Fu et al., 2003; Gaetani et al., 2003). The same authors have demonstrated that palmitoylethanolamide directly activated PPAR-α and that this lipid did not elicit anti-inflammatory effects in mutant PPAR-α-null mice (PPAR-$\alpha^{-/-}$ mice; Lo

Verme et al., 2005b). This evidence points out that the PPAR-α receptor could be a target for the palmitoylethanolamide biological actions. Importantly, it has been reported that the administration of palmitoylethanolamide decreases the endogenous levels of anandamide (Lo Verme et al., 2005b); therefore, we can suggest that palmitoylethanolamide is increasing waking via the diminution of the endogenous levels of anandamide.

Given the unexpected results in the sleep studies after palmitoylethanolamide injection, this phenomenon requires an alternative explanation. For example, it might be possible that the calcium (Ca^{2+}) could be linked with the waking caused by palmitoylethanolamide. It is noteworthy that Ambrosini et al. (2005) demonstrated that palmitoylethanolamide increased Ca^{2+} influx. Additionally, the endogenous levels of palmitoylethanolamide are increased after cerebral ischemia (Franklin et al., 2003) leading to an increase in Ca^{2+} (for a review, see Pisani et al., 2004; Halestrap, 2006). We could hypothesize that Ca^{2+} might be involved in the waking caused by palmitoylethanolamide since diverse groups have reported that Ca^{2+} is linked with the promotion of waking via the glutamatergic system (Azuma et al., 1996; Jones, 2003; Lena et al., 2005). The enhanced waking caused by palmitoylethanolamide was an unexpected and unique observation. Collectively, these results raise the provocative possibility that Ca^{2+} and glutamate may represent a novel target for palmitoylethanolamide activity. The exact comprehension of the mechanisms involved needs further investigation.

At this point, we can provide a wider scenario suggesting that oleoylethanolamide or palmitoylethanolamide could be modulating sleep–wake centers. Our group has recently reported that endogenous levels of oleoylethanolamide and palmitoylethanolamide are higher in pons and hypothalamus during the lights-off period, the active phase of rats (Murillo-Rodríguez et al., 2006a). Structures mentioned above participate importantly in the modulation of the sleep–wake cycle (Aston-Jones, 2005; Jones, 2005).

In the present study we have showed that a possible pathway activated by URB597, oleoylethanolamide and palmitoylethanolamide involves neurons in the hypothalamus and dorsal raphe nucleus showed in the c-Fos expression studies. The induction of Fos protein encoded by the immediate early gene c-Fos, is often used as a marker of neural activation. All compounds induced changes in the Fos-immunoreactive cells in some waking-related brain areas, including hypothalamic nuclei (Fig. 4) and dorsal raphe nucleus (Fig. 5). The hypothalamic nuclei have been associated with wakefulness (Sallanon et al., 1986; Suntsova et al., 2000; Suntsova and Dergacheva, 2003). For example, the dorsomedial hypothalamic nucleus contains neurons that are specifically active during wakefulness (Lin et al., 1989).

On the other hand, as shown in Fig. 5, compared to controls a population of neurons in the dorsal raphe nucleus exhibited c-Fos-immunoreactivity after URB597, oleoylethanolamide or palmitoylethanolamide microinjection. The dorsal raphe nucleus firing activity is higher in the waking state and decreases during sleep, being virtually absent in REM sleep (McGinty and Harper, 1976; Trulson and Jacobs, 1979; Jones, 2005). These findings emphasize the hypothesis that one of the neurochemical mechanisms underlying the vigilance-promoting action of URB597, oleoylethanolamide and palmitoylethanolamide could be related to its ability to enhance the serotoninergic transmission. Supporting this idea, Gobbi et al. (2005) reported that URB597 increased the firing activity of serotonergic neurons in the dorsal raphe nucleus.

Finally, dopamine levels were found enhanced after the injection of either URB597 (10 μg/5 μl, i.c.v.), oleoylethanolamide (10 μg/5 μl, i.c.v.) or palmitoylethanolamide (10 μg/5 μl, i.c.v.; Fig. 6C and D) whereas these compounds separately induced a significant decrease in L-DOPA contents (Fig. 6 C and E). In both cases, the effects were observed within a window time of 1–3 h.

We believe that the wake-inducing effects of URB597, oleoylethanolamide and palmitoylethanolamide might be associated with the increased dopamine release since lesions of dopaminergic cell groups induce a reduction in arousal in rats (Jones et al., 1973) as well as in Parkinson's disease patients (Rye and Jankovic, 2002; Paus et al., 2003).

Despite the lack of evidence on the molecular mechanism of URB597, oleoylethanolamide and palmitoylethanolamide, our study suggests a possible molecular and biochemical pathway which might be responsible for the modulation of the sleep–wake cycle by these compounds. We have described here that URB597, oleoylethanolamide and palmitoylethanolamide are inducing wakefulness by activating neurons in the hypothalamus and dorsal raphe nucleus. Since the inhibition of the FAAH via URB597 leads to an intracellular enhancement of oleoylethanolamide and palmitoylethanolamide, we hypothesized that behavioral effects observed after URB597 injection could be due to an endogenous accumulation of these lipids. Indeed, i.c.v. administrations of oleoylethanolamide or palmitoylethanolamide mimicked the effects caused by URB597 on sleep and on the dopamine levels. Although we are showing important data about the pharmacological effects of oleoylethanolamide and palmitoylethanolamide on sleep, further studies are required to determinate the molecular mechanism activated by these endogenous lipids.

The present studies further highlight the importance of the endocannabinoid system as a pharmacological target for the treatment of sleep disorders such as excessive somnolence. This sleep disturbance is defined as trouble falling asleep or staying asleep and can cause feeling sleepiness or fatigue during the day, affect mood, and result in trouble focusing on tasks. According to the National Sleep Foundation results from the 2005 *Sleep in America* poll indicate that 60% of America's adults who drive or have a license report that, within the past year, they have driven a car or motor vehicle when feeling drowsy. Regardless, continued investigations into each of these compounds searching for the molecular mechanism that they might activate to modulate the sleep–wake cycle should greatly enrich our understanding of the physiological functions of the endocannabinoid system.

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