REVEALING THE ROLE OF THE ENDOCANNABINOID SYSTEM MODULATORS, SR141716A, URB597 AND VDM-11, IN SLEEP HOMEOSTASIS

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Abstract—The endocannabinoid system comprises receptors (CB_1 and CB_2 cannabinoid receptors), enzymes (Fatty Acid Amide Hydrolase [FAAH], which synthesizes the endocannabinoid anandamide), as well as the anandamide membrane transporter (AMT). Importantly, previous experiments have demonstrated that the endocannabinoid system modulates multiple neurobiological functions, including sleep. For instance, SR141716A (the CB_1 cannabinoid receptor antagonist) as well as URB597 (the FAAH inhibitor) increase waking in rats whereas VDM-11 (the blocker of the AMT) enhances sleep in rodents. However, no further evidence is available regarding the neurobiological role of the endocannabinoid system in the homeostatic control of sleep. Therefore, the aim of the current experiment was to test if SR141716A, URB597 or VDM-11 would modulate the sleep rebound after sleep deprivation. Thus, these compounds were systemically injected (5, 10, 20 mg/kg; ip; separately each one) into rats after prolonged waking. We found that SR141716A and URB597 blocked in dose-dependent fashion the sleep rebound whereas animals treated with VDM-11 displayed sleep rebound during the recovery period. Complementary, injection after sleep deprivation of either SR141716A or URB597 enhanced dose-dependently the extracellular levels of dopamine (DA), norepinephrine (NE), epinephrine (EP), serotonin (5-HT), as well as adenosine (AD) while VDM-11 caused a decline in contents of these molecules. These findings suggest that SR141716A or URB597 behave as a potent stimulants since they suppressed the sleep recovery period after prolonged waking. It can be concluded that elements of the endocannabinoid system, such as the CB_1 cannabinoid receptor, FAAH and AMT, modulate the sleep homeostasis after prolonged waking.

Key words: alertness, sleep deprivation, microdialysis, REM sleep, serotonin, HPLC.

INTRODUCTION

The neurobiological actions of endogenous cannabinoid-like compounds have been described from molecular to behavioral approaches (Pertwee, 2006, 2015; Scherma et al., 2014; Katona, 2015; Lutz et al., 2015; Manduca et al., 2015; Velasco et al., 2015; Lu and Mackie, 2016; Nikan et al., 2016; Wang et al., 2016). Since anandamide was the very first endocannabinoid described (Devane et al., 1992), it has been the focus of attention due to that it resembles the pharmacological effects of principal compound of *Cannabis sativa*, Δ^9-THC, by behaving as a natural ligand for the CB_1 cannabinoid receptor (Console-Bram et al., 2012). Among the evidence regarding the role of activation of the CB_1 cannabinoid receptor modulating diverse functions controlled by the central nervous system, it has been demonstrated that administrations of anandamide promote sleep (Murillo-Rodríguez et al., 1998, 2001, 2003; Herrera-Solis et al., 2010; Rueda-Orozco et al., 2010), whereas injections of the CB_1 cannabinoid receptor antagonist SR141716A

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Abbreviations: 5-HT, serotonin; AcbC, nucleus accumbens; AD, adenosine; AMT, anandamide membrane transporter; aCSF, artificial cerebrospinal fluid; DA, dopamine; EEG, electroencephalogram; EMG, electromyogram; EP, epinephrine; FAAH, Fatty Acid Amide Hydrolase; HPLC, high-performance liquid chromatography; NE, norepinephrine; REMS, rapid eye movement sleep; SWS, slow wave sleep; TSD, total sleep deprivation; VEH, vehicle solution; W, wakefulness.

increase alertness in rodents (Santucci et al., 1996; Murillo-Rodríguez et al., 2001, 2003).

To elucidate the neurobiological properties of other elements belonging to the endocannabinoid system, such as anandamide's major metabolic enzyme Fatty Acid Amide Hydrolase (FAAH), several studies have been aimed to describe the effects of FAAH in multiple experimental paradigms (Chauvet et al., 2015; Fowler, 2015; Panlilio et al., 2016), including experiments in sleep. For instance, FAAH-KO mice display no significant differences in sleep rebound after prolonged waking sessions (Huitrón-Reséndiz et al., 2004) whereas studies outline that levels of expression of FAAH are lower in the brain after sleep deprivation in naïve animals (Wang et al., 2011). In addition, pharmacological experiments using the FAAH inhibitor named URB597, have shown that injections of this compound enhance wakefulness (W) and decrease slow wave sleep (SWS) as well as rapid eye movement sleep (REMS) in rats (Murillo-Rodríguez et al., 2007, 2011).

An additional actor of the endocannabinoid system includes the anandamide membrane transporter (AMT) which displays a key role in modulating anandamide's biological functions (Chicca et al., 2012; Fowler, 2013; Khasabova et al., 2013; Leung et al., 2013; Nicolussi and Gertsch, 2015). The available evidence indicates that injections of blocker of AMT known as VDM-11 promote sleep in rats (Murillo-Rodríguez et al., 2008, 2013). To sum up, convincing available data suggest that CB_1 cannabinoid receptors, FAAH and AMT may contribute to sleep control. However, the role of these molecular mediators in the regulation of sleep homeostasis has been scantly described so far.

In the last decades, accumulating reports have suggested that the sleep–wake cycle is under control of a complex neurobiological network which includes neuroanatomical, neurochemical and molecular components (Murillo-Rodríguez et al., 2012; Zeitzer, 2013; Saper, 2013; Fernandes et al., 2015; Schwartz and Kilduff, 2015). In parallel, further complicatedness in the understanding of sleep mechanism has been pointed-out due to that circadian and homeostatic factors influence sleep processes (Curie et al., 2013, 2015; Houben et al., 2014; Borbely et al., 2016). In regard to the homeostatic clout in sleep modulation and consistent with previous reports, it is known that a compensatory enhancement in the intensity and duration of sleep known as ''sleep rebound" is present as a result of significative loss of sleep (Borbely and Achermann, 1999; Schwierin et al., 1999; Ocampo-Garces et al., 2000; Vyazovskiy et al., 2007, 2011; Borbely et al., 2016). Additionally, the total sleep deprivation (TSD) is accompanied with an accumulation of endogenous neurochemicals, including dopamine (DA), norepinephrine (NE), epinephrine (EP), serotonin (5-HT), as well as adenosine (AD). As part of the sleep homeostasis, animals that are allowed to sleep once TSD is over show a diminishing profile in the contents of several neurochemicals (Porkka-Heiskanen et al., 2000, 2002; Lena et al., 2005; Zant et al., 2011; Brown et al., 2012). In spite of the findings of the effects of the endocannabinoids in sleep modulation, no evidence is available regarding its influence in sleep homeostasis control. Thus, in particular, it is important to assess whether SR141716A, URB597 or VDM-11 would modulate sleep homeostasis as well as neurochemicals related with sleep after prolonged waking.

EXPERIMENTAL PROCEDURES

Experiment 1: blockade of sleep rebound by SR141716A, URB597 or VDM-11 injection after total sleep deprivation

Animals. Male Wistar rats (n = 20; 250–300 g) were singly housed in polycarbonate cages (48.26 cm × 26.67 cm × 20.32 cm; Harlan Laboratories. México) under light–dark cycle (lights-on: 07:00–19:00 h), humidity controlled (60 ± 10%) and constant temperature (21 ± 1 °C). In all time, rats had free access to Purina Rat Chow (Purina. México) as well as tap water. The experimental protocols were approved by the Research and Ethics Committee of our University fulfilling the domestic and international standards of animal welfare including the Mexican Standards Related to Use and Management of Laboratory Animals (DOF. NOM-062-Z00-1999) as well as the National Institutes of Health (NIH publication No. 80-23, revised 1996). During the whole study, efforts to minimize animal suffering were considered and for ethical reasons, it used a reduced number of animals.

Chemicals. Compounds such as SR141716A (CAS Number: 158681-13-1), URB597 (CAS Number 546141-08-6) and VDM-11 (CAS Number 313998-81-1) were purchased from Sigma–Aldrich (St Louis, MO. USA). These chemicals were dissolved in a vehicle (VEH) solution composed of polyethylglycol/saline (5:95 v/v) as described previously (Murillo-Rodríguez et al., 2003, 2011, 2013). All reagents, chemicals, and materials were purchased from Sigma–Aldrich (St Louis, MO. USA).

Sleep-recording surgeries. Animals (n = 10) were anesthetized by a mixture of acepromazine (0.75 mg/kg), xylazine (2.5 mg/kg), and ketamine (22 mg/kg) and placed in a stereotaxic frame (David Kopf Instruments. Tujunga, CA. USA) for sleep-recording electrodes implantation. Two stainless-steel screw electrodes were inserted 2 mm on either side of the sagittal sinus and 3 mm anterior to Bregma (frontal cortex) whereas other two screws were located 3 mm on either side of the sagittal sinus and 6 mm behind Bregma (occipital cortex). This setting recorded the electroencephalogram (EEG) signal by the bipolar (differential) EEG recorded from two contralateral screw electrodes (frontal–occipital). In regard to the recording of the electromyogram (EMG), this signal was obtained by the implantation of two wire electrodes into the dorsal neck muscles in each rat. Next, EEG/EMG wires were inserted into a six-pin plastic plug (Plastics One. Roanoke, VA. USA) and they were secured onto the skull by commercial dental cement. Upon completion of the EEG/EMG surgeries, the rats were placed into

individual cages with regular bedding and standard water and food containers. Animals were connected to a 6-channel slip-ring commutator through a 50-cm cable (Plastics One. Roanoke, VA. USA), which allowed rats free turning and moving around in the box during post-operative recovery period. The surgical procedure as well as habituation conditions (during 7 days) were carried out as reported elsewhere (Murillo-Rodríguez et al., 2011).

Sleep recordings analysis. The EEG/EMG data were scored in 12 s epochs characterizing W, SWS or REMS by the sleep-scoring program (ICELUS). Briefly, the EEG/EMG wires were connected to the slip-ring system (Plastics One. Roanoke, VA. USA) and to the amplifier which filtered the EEG signal at 70 Hz (low-pass filter) and 0.3 Hz (high-pass filter; Model M15LT 15A54; Grass Instruments. Quincy, MA. USA). The signals were continuously sampled at 128 Hz using a 100-bit analog-to-digital converter board (NI PCI-6033E Multifunction I/O Board and NI-DAQ Software, SCB-100 Shielded Connector Block. National Instruments. Austin, TX. USA). The sleep–wake cycle was characterized as follows: The waking state was identified by the presence of desynchronized EEG and high EMG activity whereas SWS consisted of high-amplitude slow waves together with a low EMG tone relative to awake. Lastly, REMS was determined by the presence of regular theta activity coupled with low EMG relative to SWS. The entire sleep-recordings analysis procedure was followed as previously reported (Mijangos-Moreno et al., 2016). Immediately after 6 h of TSD, experimental treatments were given to rats and sleep data were obtained during the following 4 h and analyzed as previously reported (Murillo-Rodríguez et al., 2011, 2013).

Total sleep-deprivation. Prolonged waking was carried out by maintaining rats on constant alertness across 6 h (from 07:00–13:00 h) by stroking fur with an artist's paintbrush, provoking light noise near the cages or by tapping or placing novel objects into the cages (Arias-Carrión et al., 2011). During the TSD, sleep data were constantly recorded and analyzed as reported (Murillo-Rodríguez et al., 2011).

Pharmacological administrations. Right after the end of TSD, animals were disconnected from the sleep-recording system and experimental trials were administered during the lights-on period randomly as follows: VEH, SR141716A, URB597 or VDM-11 (5, 10, 20 mg/kg, i.p.; $n = 10$ each group). Irrespective of whether the injection of VEH could modify the sleep–wake cycle, an additional group named "sham" ($n = 10$) was included in the experimental design. This group consisted in animals that received the insertion (i.p.) of the needle but no administration was given. To avoid possible accumulative pharmacological effects, animals were under a "wash out period" which consisted in 24 h period between treatments. Once experimental challenges were given, rats were reattached to the sleep-recording system and sleep data were collected across the next 4 h. Sleep stages were obtained across 4 h (hour-1, hour-2, hour-3 and hour-4) post-treatments during the sleep recovery period. Next, we averaged the four time points as a total value for each state of vigilance (W, SWS or REMS) in every experimental condition. Then, these total values were included in the corresponding statistical analysis. Lastly, in the current experiment, treatments were done randomly using a single-blind Latin Square Experimental Design. Since each subject serves as its own control, the testing paradigm has the advantage of decreasing variability among experimental conditions.

Statistical analysis. Data were represented as mean ± standard error of the mean. Statistical differences were determined by a one-way ANOVA followed by Scheffé's post hoc test for multiple comparisons. All statistical analyses were performed using the StatView (version 5.0.0, SAS Institute. USA) and statistical differences among groups were determined if $P < 0.05$.

Experiment 2: effects of SR141716A, URB597 or VDM-11 on neurochemical levels during the sleep rebound after 6 h of prolonged waking

Animals. A new set of rats was employed for this part of the study. All animals were housed as described in Experiment 1.

Chemicals. All compounds as described in Experiment 1.

Microdialysis surgeries. Experiments have demonstrated the critical role of nucleus accumbens (AcbC) in sleep control (Lazarus et al., 2012, 2013; Qiu et al., 2012; Zhang et al., 2013; Liu et al., 2016). Moreover, previous work from our laboratory has provided reliable measurements of monoamines and AD from AcbC (Murillo-Rodríguez et al., 2006; Mijangos-Moreno et al., 2014, 2016). Thus, these previous evidence allowed us to consider AcbC as candidate for microdialysis sampling. To achieve this goal, a new set of animals ($n = 10$) was anesthetized and placed into the stereotaxic frame (David Kopf Instruments. Tujunga, CA. USA) for implantation of a microdialysis guide-cannula (IC guide; BioAnalytical Systems [BAS]. West Lafayette, IN. USA) which was unilaterally placed into the AcbC (coordinates: A = +1.2 and L = +2.0, H = −7.0 mm, with reference to Bregma [Paxinos and Watson, 2005]). After the surgery, animals were placed individually into the microdialysis bowl (Raturn Microdialysis Stand-Alone System. MD-1404. BAS. West Lafayette, IN. USA) for recovery as well as habituation for the experimental conditions during 7 days. All surgical procedures were accomplished as previously reported (Mijangos-Moreno et al., 2014, 2016).

Microdialysis sampling procedures. One week after microdialysis surgery the stylet from guide-cannula was withdrawn and microdialysis probe (1 mm of length; polyacrylonitrile, MWCO = 30,000 Daltons; 340 μm OD; BAS. West Lafayette, IN. USA) was inserted at 07:00 h.

Artificial cerebrospinal fluid (aCSF [composition: NaCl 147 mM, KCl 3 mM, CaCl 1.2 mM, MgCl 1.0 mM, pH 7.2]) was continuously perfused through a minitube (0.65 mm OD × 0.12 mm ID; BAS. West Lafayette, IN. USA) attached to a 2.5 mL syringe (BAS. West Lafayette, IN. USA) using a pump (flow rate: 0.25 μL/min; BAS Bee. West Lafayette, IN, USA). On the other hand, previous experiments have found that after insertion, the microdialysis probe requires 6–7 h for stabilization (Porkka-Heiskanen et al., 2000; Murillo-Rodríguez et al., 2004), in the present report, the probes were stabilized during 24 h in freely moving rats. Once stabilization period was achieved and right after the sleep deprivation period, microdialysis samples were collected hourly from each animal starting at 13:00 h across the lights-on period over 4 h (dialysates were collected the first 20 min of every hour). Next, samples were averaged over the four time points as a total value for each compound in every experimental condition (sham, VEH, SR141716A, URB597 and VDM-11; separately all doses for each drug). Thus, these total values were included in the corresponding statistical analysis. Therefore, the reported data represent the average of hourly concentration over a period of time of 4 h of sampling from each molecule. However, an example of the time course, of at least one of the neuromodulators, during the total sleep deprivation (six samples taken at hourly intervals) and the sleep recovery period (four samples taken at hourly intervals) was analyzed. In addition, the microdialysis probes were used no more than 5 days since precedent studies have reported that subsequently insertion, gliosis diminishes the membrane ability to transport fluid across the pores (Porkka-Heiskanen et al., 2000; Murillo-Rodríguez et al., 2004). For the *in vitro* recovery data, aCSF solution was perfused at the flow rate of 0.25 μL/min into the microdialysis probe, which was inserted into the test solution containing external known concentrations of monoamines or AD. The same sampling procedure was repeated using a different lot of microdialysis probes. Later, each sample collected from *in vitro* recovery study was analyzed and the peak area ratio was calculated against the known standards. To obtain the recovery rate, data were calculated as follows: Recovery rate (%) = (the peak area ratio of the sample from microdialysis sample)/(the peak area ratio of the sample in the test solution). Further details of microdialysis procedures are available in previous reports (Porkka-Heiskanen et al., 2000; Blanco-Centurion et al., 2006; Murillo-Rodríguez et al., 2007, 2011; Mijangos-Moreno et al., 2014, 2016).

Sleep deprivation procedure. As described in Experiment 1.

Pharmacological challenge. Twenty-four hours after stabilization of microdialysis probes, TSD was carried out from 07:00 to 13:00 h. Once the prolonged waking session finished, animals were disconnected from microdialysis system and treatments were randomly given during the lights-on period as described in Experiment 1. Right after the experimental challenges were provided, animals were reattached to the microdialysis system and samples were collected across 4 h and stored (−80 °C) for further analysis.

High-performance liquid chromatography (HPLC). The collected dialysates were injected into HPLC for detection and quantification of DA, NE, 5-HT or AD. Briefly, the dialysates were filtered (Millipore 0.22 μm; Merck Millipore. Darmstadt, Germany) and injected into HPLC (Modular Prominence, Shimadzu. Japan). The mobile phase (monosodium phosphate [7 mM, pH 3.0], plus methanol [3.5%]) was perfused at a flow rate of 80 μL/min using a pump (LC-20AT, Shimadzu, Japan) and the separation of molecules was achieved using a microbore column (octadecyl silica [3 μm, 100 × 1 mm]. BAS, West Lafayette, IN, USA). To further determine the contents of DA, NE, 5-HT or AD in the microdialysis samples, a series of dilutions of known external standards for monoamines or AD were prepared. The method to ensure the specificity was developed following the instructions provided by the manufacturer as well as reported by others (Sartori et al., 2008; Mazzucchelli et al., 2011). Detection of monoamines carried out using an electrochemical detector (LC-4C; BAS. West Lafayette. IN, USA) as described by others (Gold et al., 2011; Grupe et al., 2013) whereas AD was detected using an UV detector (SPD-20A, Shimadzu. Japan) set to a wavelength of 254 nm (deuterium lamp). All chromatographic data were recorded in a personal computer, and peak heights of neurochemicals in dialysates were compared with external standards using chromatograph report software (LC Solution, Shimadzu. Japan). All procedures for detection and measurement of monoamines or AD were developed according to previous reports (Murillo-Rodríguez et al., 2011; Mijangos-Moreno et al., 2014, 2016).

Histological verification of probe location. After microdialysis experiments, all animals were anesthetized with a lethal dose of pentobarbital for the standard procedure for the vascular perfusion. Rats were transcardially perfused through a ventricular catheter placed on the left ventricle. Next, the right atrium was cut open and saline solution (0.9%) followed by formaldehyde (4%) were perfused. The brain was removed and post-fixed overnight in formaldehyde (4%) followed by 30% sucrose/0.1 M PBS for 48 h. Last, all brains were cut in coronal sections (20 μm) using a Portable Bench-top Cryostat (Leica CM1100. Germany) and collected in 1:5 serial order. One serial was used for probe location and it was identified by plotting using rat brain atlas (Paxinos and Watson, 2005).

Statistical analysis. Data from microdialysis experiments were represented as mean ± standard error of the mean. The statistical analyses were performed by StatView program (version 5.0.0, SAS Institute, USA) and statistical significances were determined by a one-way ANOVA followed by Scheffé's post hoc test for multiple comparisons. Values of $P < 0.05$ were considered as statistically significant.

RESULTS

Experiment 1: blockade of sleep rebound by SR141716A, URB597 or VDM-11 injection after total sleep deprivation

As expected, no statistical differences were found between sham and VEH groups in total time of W, SWS or REMS after TSD (Panel A, Panel B, Panel C, respectively). However, it was found that during the sleep rebound period, and compared to respective control group, administrations of SR141716A (5, 10, 20 mg/kg) caused a dose-dependent enhancement in alertness (Fig. 1, Panel A; $F_{(4, 45)} = 2630.75$; $P < 0.0001$) as well as a decrease in SWS (Fig. 1, Panel A; $F_{(4, 45)} = 453.69$; $P < 0.0001$) and REMS (Fig. 1, Panel A; $F_{(4, 45)} = 120.35$; $P < 0.0001$). It was found that SR141716A behaved as a potent stimulant since promoted alertness during the sleep rebound period after 6 h of prolonged waking. Similar, but not identical findings were observed comparing to control/sham rats in URB-treated animals (5, 10, 20 mg/kg) since a blocking effect in the sleep rebound was observed by increasing alertness (Fig. 1, Panel B; $F_{(4, 45)} = 175.262$; $P < 0.0001$) and diminishing in a dose-dependent fashion SWS (Fig. 1, Panel B; $F_{(4, 45)} = 102.790$; $P < 0.0001$) and REMS (Fig. 1, Panel B; $F_{(4, 45)} = 356.884$; $P < 0.0001$). Despite these fascinating results, URB597 was not able to induce a significant dose-dependent effect in W and REMS. Moreover, it is noteworthy that this compound blocked the sleep rebound of either SWS or REMS after 6 h of TSD in more potent fashion that SR141716A. This data indicate the effectiveness of URB597 by promoting W after sleep deprivation. Reversed results were found when VDM-11 (5, 10, 20 mg/kg) was injected in sleep-deprived rats. For instance, no differences were found among control/sham groups in W (Fig. 1, Panel C; $F_{(4, 45)} = 0.00$; $P > 0.05$), SWS (Fig. 1, Panel C; $F_{(4, 45)} = 3.191$; $P > 0.05$) as well as REMS (Fig. 1, Panel C; $F_{(4, 45)} = 4.493$; $P > 0.05$). This drug did not block the sleep rebound in rats subjected to TSD. Taken together, our data suggest that the CB_1 cannabinoid receptor, FAAH and AMT play a role in the modulation of sleep homeostasis after sleep deprivation.

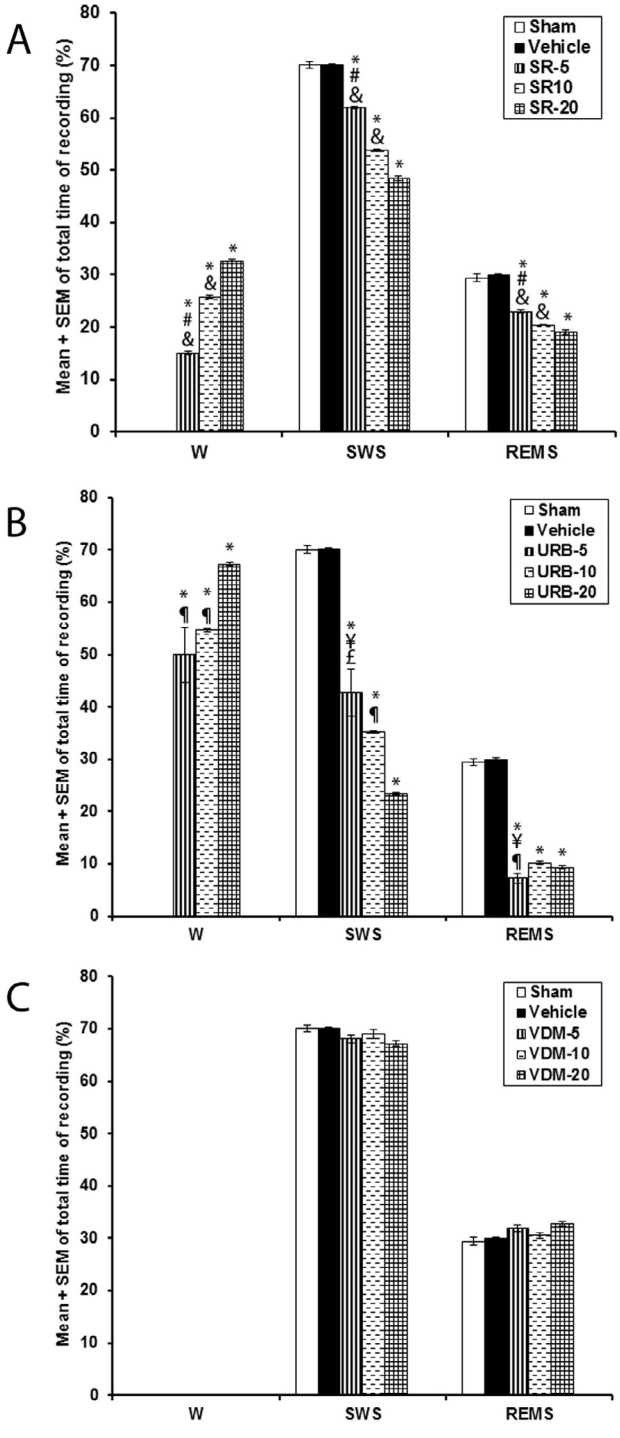

Fig. 1. Effects of pharmacological manipulation of the endocannabinoid system in wakefulness (W), slow wave sleep (SWS) or rapid eye movement (REMS) during the sleep rebound period in sleep-deprived rats. Panel A shows the effects of administration of SR141716A (10, 20 or 30 mg/kg, i.p.) after total sleep deprivation period. As noted, SR141716A enhanced in dose-dependent fashion W ($F_{(4, 45)} = 2630.75$; $P > 0.0001$) and decreased SWS ($F_{(4, 45)} = 453.69$; $P > 0.0001$) and REMS ($F_{(4, 45)} = 120.35$; $P > 0.0001$; * vs Control/VEH, $P < 0.0001$; # vs SR-10 (SR141716A, 10 mg/kg, i.p.), & vs SR-20 (SR141716A, 20 mg/kg, i.p.). On the other hand, the effects on sleep rebound of FAAH inhibition using URB597 (10, 20 or 30 mg/kg, i.p.) are shown in Panel B. As indicated, URB597 blocked the sleep rebound by enhancing W ($F_{(4, 45)} = 175.262$; $P > 0.0001$) and diminishing SWS ($F_{(4, 45)} = 102.790$; $P > 0.0001$) and REMS ($F_{(4, 45)} = 356.884$; $P > 0.0001$) during the sleep rebound period (*vs Control/VEH, $P < 0.0001$; ¥ vs URB-10 (URB597, 10 mg/kg, i.p.), ¶ vs URB-20 (URB597, 20 mg/kg, i.p.). Lastly, the administration of VDM-11 (10, 20 or 30 mg/kg, i.p.) after total sleep deprivation caused no statistical effects on W ($F_{(4, 45)} = 0.00$; $P > 0.05$), SWS ($F_{(4, 45)} = 3.191$; $P > 0.05$) or REMS ($F_{(4, 45)} = 4.493$; $P > 0.05$) during the sleep rebound period. All comparisons determined by a one-way ANOVA followed by Scheffés post hoc where $P < 0.001$ considered statistically different.

Experiment 2: effects of SR141716A, URB597 or VDM-11 on neurochemical levels during the sleep rebound after 6 h of prolonged waking

It is widely known that prolonged waking induces endogenous accumulation of several neurochemicals such as monoamines and purines whereas during the

sleep rebound period, the contents of these molecules are decreased (Porkka-Heiskanen et al., 2000, 2002; Lena et al., 2005; Blanco-Centurion et al., 2006; Monti and Jantos, 2008; Mohammed et al., 2011; Brown et al., 2012). Thus, the rationale of the following experiment was based to test whether SR141716A, URB597 or VDM-11 would modulate the extracellular contents of DA, NE, EP, 5-HT or AD after prolonged waking. We assessed the effects of the drugs on contents of these neurochemicals after sleep deprivation by microdialysis and HPLC means. First, levels of DA were not statistically different during TSD among groups (Fig. 2, Panel A; $F_{(4,\ 45)} = 0.587$; $P > 0.05$). However, during sleep rebound period and compared to control/sham groups, animals that received SR141716A promoted the endogenous accumulation of DA in a dose-dependent fashion (Fig. 2, Panel B; $F_{(4,\ 45)} = 220.725$; $P < 0.0001$). Moreover, injections of URB597 induced a dose-dependent enhancement in the contents of DA (Fig. 2, Panel C; $F_{(4,\ 45)} = 1951.791$; $P < 0.0001$). Nonetheless, administrations of VDM-11 at different doses (5, 10 or 20 mg/kg, i.p.) decreased the contents of DA (Fig. 2, Panel D; $F_{(4,\ 45)} = 82.179$; $P < 0.0001$). Comparing the pharmacological effects of the compounds tested after TSD, we conclude that

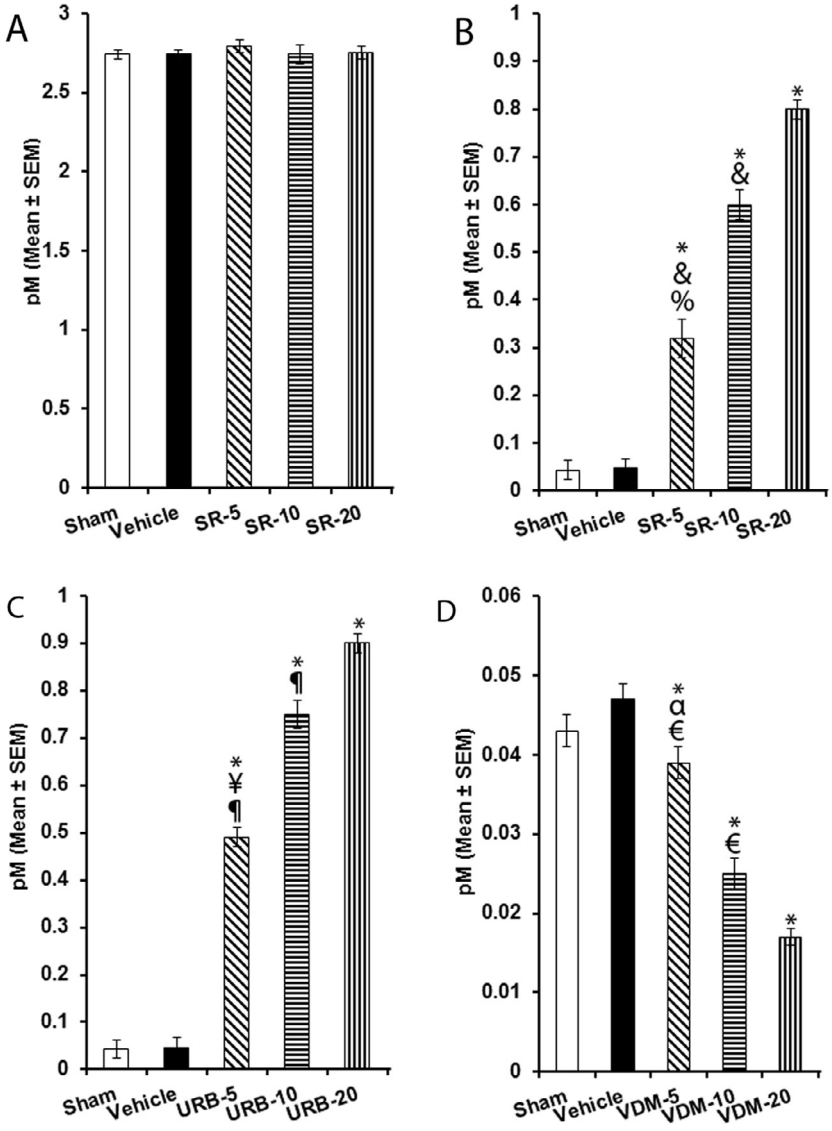

Fig. 2. Effects of pharmacological activation or blockade of the endocannabinoid system in the extracellular levels of dopamine in response to 6 h of prolonged waking. Levels of dopamine were enhanced after 6 h of prolonged waking (Panel A; $F_{(4,\ 45)} = 0.587$; $P > 0.05$). Next, rats received the administration of either vehicle, SR141716A, URB597 or VDM-11 after prolonged waking (10, 20 or 30 mg/kg; i.p. each compound) and dopamine contents were analyzed. Panel B shows that SR141716A enhanced in a dose-dependent fashion the levels of dopamine ($F_{(4,\ 45)} = 220.725$; $P < 0.0001$;* vs Control/VEH, # vs SR-10 (SR141716A, 10 mg/kg, i.p.), & vs SR-20 (SR141716A, 20 mg/kg, i.p.). Similar results were observed after injection of URB597 (Panel C; $F_{(4,\ 45)} = 1951.791$; $P < 0.0001$* vs Control/VEH, ¥ vs URB-10 (URB597, 10 mg/kg, i.p.), ¶ vs URB-20 (URB597, 20 mg/kg, i.p.). As indicated in Panel D, VDM-11 promoted a diminution in contents of dopamine ($F_{(4,\ 45)} = 82.179$; $P < 0.0001$;* vs Control/VEH, α vs VDM-10 (VDM-11, 10 mg/kg, i.p.), € vs VDM-20 (VDM-11, 20 mg/kg, i.p.). All comparisons determined by a one-way ANOVA followed by Scheffé's post hoc where $P < 0.001$ considered statistically different.

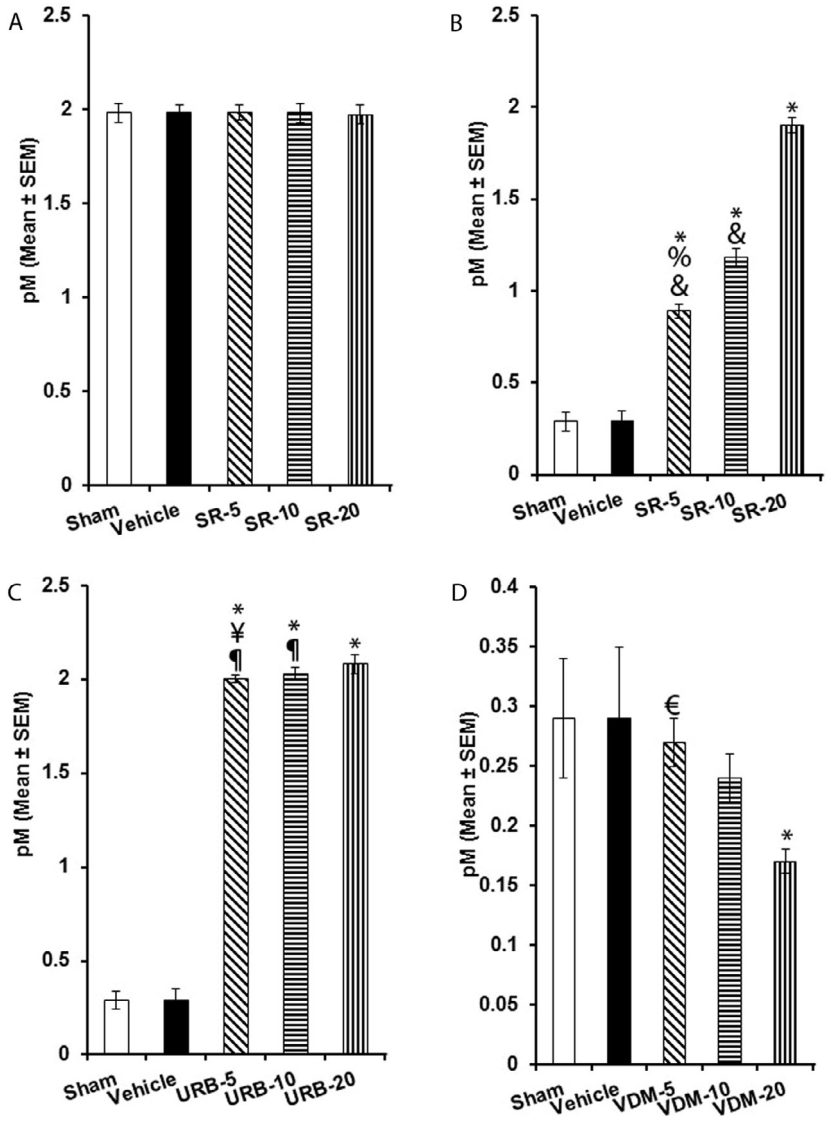

Fig. 3. Effects of SR141716A, URB597 or VDM-11 (10, 20 or 30 mg/kg; i.p. each compound) on extracellular levels of norepinephrine after 6 h of prolonged waking. Panel A shows that after 6 h or total sleep deprivation, contents of norepinephrine were enhanced ($F_{(4,45)} = 0.521$; $P < 0.05$). When animals received SR141716A immediately after prolonged waking, it was found an increase in a dose-dependent fashion the contents of norepinephrine (Panel B; $F_{(4, 45)} = 15258.779$; $P < 0.0001$;* vs Control/VEH, # vs SR-10 (SR141716A, 10 mg/kg, i.p.), & vs SR-20 (SR141716A, 20 mg/kg, i.p.). URB597 caused similar results (Panel C; $F_{(4,4\ 5)} = 29135.400$; $P < 0.0001$;* vs Control/VEH, ¥ vs URB-10 (URB597, 10 mg/kg, i. p.), ¶ vs URB-20 (URB597, 20 mg/kg, i.p.). Finally, injections of VDM-11 decreased the levels of norepinephrine (Panel D; $F_{(4, 45)} = 7.185$; $P < 0.0001$;* vs Control/VEH, α vs VDM-10 (VDM-11, 10 mg/kg, i.p.), € vs VDM-20 (VDM-11, 20 mg/kg, i.p.). All comparisons determined by a one-way ANOVA followed by Scheffé's post hoc where $P < 0.001$ considered statistically different.

URB597 displayed more potent response in promotion of the endogenous accumulation of DA whereas VDM-11 induced opposite outcomes.

After injection of drugs, interesting results in the levels of NE were found during the sleep rebound period. For example, during TSD no statistical differences were found in contents of NE among groups (Fig. 3, Panel A; $F_{(4, 45)} = 0.521$; $P > 0.05$). Nevertheless, administrations of SR141716A before the sleep rebound period enhanced the levels of NE in a dose-dependent fashion (Fig. 3, Panel B; $F_{(4, 45)} = 15258.779$; $P < 0.0001$). Moreover, URB597-treated rats displayed a dose-dependent enhancement in the contents of NE (Fig. 3, Panel C; $F_{(4, 45)} = 29135.400$; $P < 0.0001$) whereas the injections of VDM-11 at different doses (5, 10 or 20 mg/kg, i.p.) decreased NE levels (Fig. 3, Panel D; $F_{(4, 45)} = 7.185$; $P < 0.0001$). It is important to mention that only the highest dose of VDM-11 decreased significantly the extracellular levels of NE. It is concluded that URB597 showed the most potent effects by increasing the accumulation of NE compared to SR141716A whereas VDM-11 promoted contrary effects.

After measuring the endogenous levels of EP during prolonged waking, we found no statistical differences among groups (Fig. 4, Panel A; $F_{(4, 45)} = 0.95$; $P > 0.05$). However, administrations of SR141716A caused a dose-dependent enhancement of contents of EP after TSD (Fig. 4, Panel B; $F_{(4, 45)} = 134.970$; $P < 0.0001$) whereas

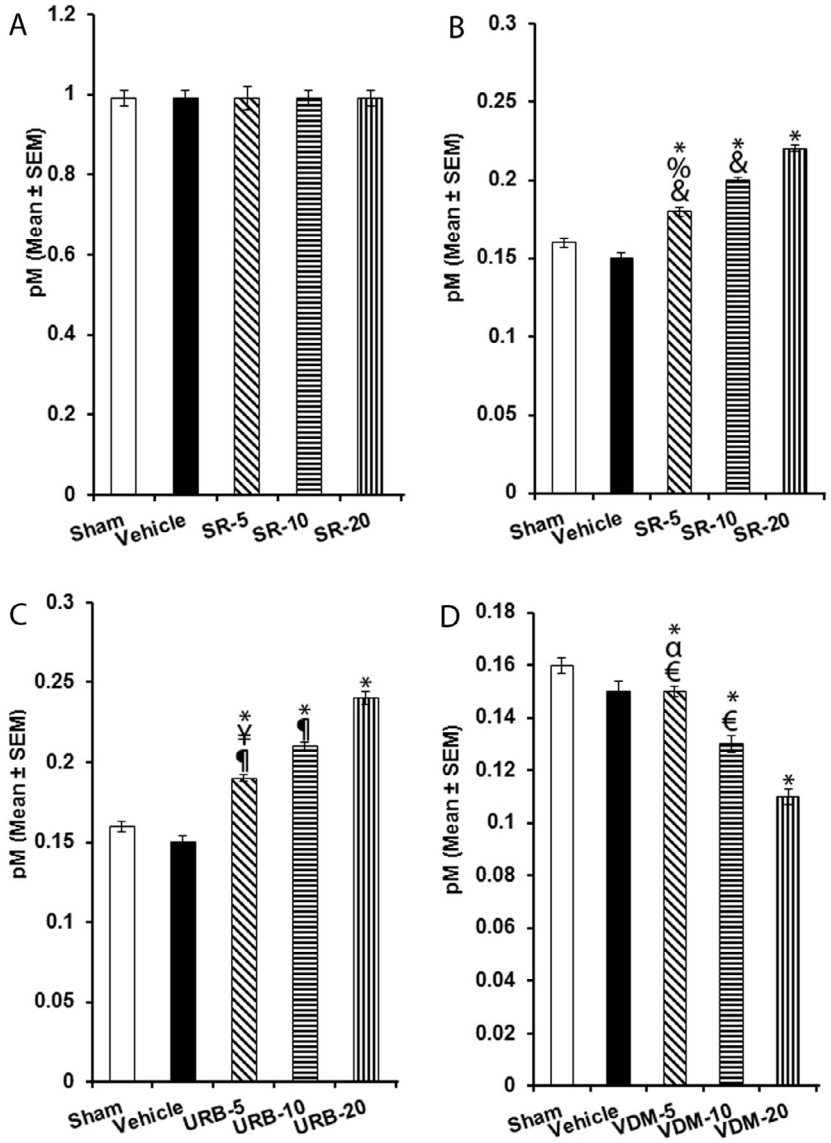

Fig. 4. Effects of SR141716A, URB597 or VDM-11 (10, 20 or 30 mg/kg; i.p. each compound) on extracellular contents of epinephrine collected by microdialysis after 6 h of prolonged waking. In response to total sleep deprivation, animals showed an increase in the extracellular levels of epinephrine after total sleep deprivation (Panel A; $F_{(4, 45)} = 0.95$; $P > 0.05$). However, when animals received SR141716A, we found a robust enhancement in epinephrine levels (Panel B; $F_{(4, 45)} = 134.970$; $P < 0.0001$;* vs Control/VEH, # vs SR-10 (SR141716A, 10 mg/kg, i.p.), & vs SR-20 (SR141716A, 20 mg/kg, i.p.). Moreover, URB597 promoted similar results (Panel C; $F_{(4,45)} = 164.245$; $P < 0.0001$;* vs Control/VEH, ¥ vs URB-10 (URB597, 10 mg/kg, i.p.), ¶ vs URB-20 (URB597, 20 mg/kg, i.p.) whereas administration of VDM-11 caused opposite effects (Panel D; $F_{(4,45)} = 36.977$; $P < 0.0001$;* vs Control/VEH, α vs VDM-10 (VDM-11, 10 mg/kg, i.p.), € vs VDM-20 (VDM-11, 20 mg/kg, i.p.). All comparisons determined by a one-way ANOVA followed by Scheffé's post hoc where $P < 0.001$ considered statistically different.

injections of URB597 caused a dose-dependent decrease in EP levels after prolonged waking (Fig. 4, Panel C; $F_{(4, 45)} = 164.245$; $P < 0.0001$). Likewise, during sleep rebound period, animals that received VDM-11 showed a diminishing effects on the extracellular contents of EP (Fig. 4, Panel D; $F_{(4, 45)} = 36.977$; $P < 0.0001$). As shown in the respective Figures, SR141716A and URB597 caused similar effects by enhancing the levels of EP during sleep rebound period whereas VDM-11 decreased significantly the contents of this neurotransmitter after prolonged waking.

Analysis of the 5-HT levels during TSD showed no statistical differences among the experimental groups (Fig. 5, Panel A; $F_{(4, 45)} = 0.592$; $P > 0.05$). Albeit fascinating results were observed since SR141716A enhanced in a dose-dependent fashion the contents of 5-HT (Fig. 5, Panel B; $F_{(4, 45)} = 1678.310$; $P < 0.0001$). Likewise, administrations of URB597 provoked a dose-dependent increase in the levels of 5-HT after TSD (Fig. 5, Panel C; $F_{(4, 45)} = 1522.333$; $P < 0.0001$). Lastly, contents of 5-HT were found diminished in a dose-dependent fashion after VDM-11 injection during sleep

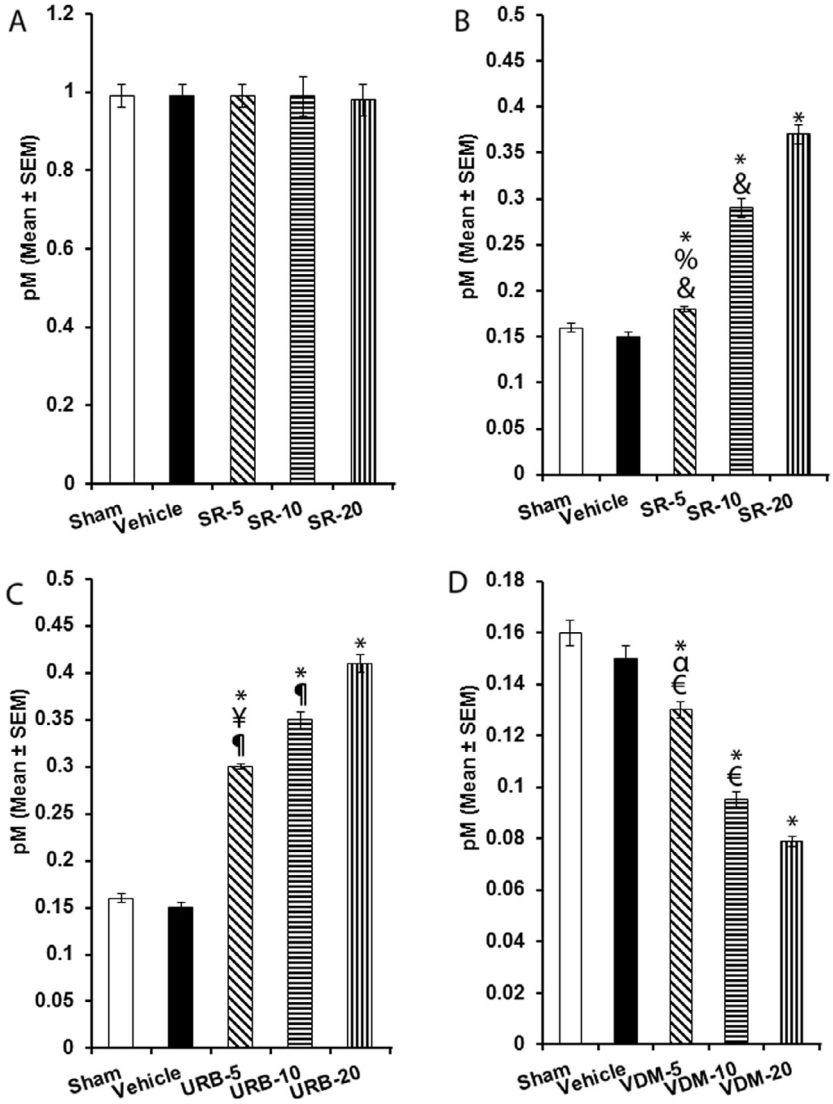

Fig. 5. Effects of SR141716A, URB597 or VDM-11 (10, 20 or 30 mg/kg; i.p. each compound) on levels of 5-HT collected after 6 h of prolonged waking. Panel A shows that extracellular levels of 5-HT were enhanced after total sleep deprivation ($F_{(4, 45)} = 0.592$; $P > 0.05$). However, when rats received SR141716A, the levels of 5-HT were increased in a dose-dependent fashion (Panel B; $F_{(4, 45)} = 1678.310$; $P < 0.0001$;* vs Control/VEH, # vs SR-10 (SR141716A, 10 mg/kg, i.p.), & vs SR-20 (SR141716A, 20 mg/kg, i.p.). Similar results were observed in URB597-treated rats (Panel C; $F_{(4, 45)} = 1522.333$; $P < 0.0001$;* vs Control/VEH, ¥ vs URB-10 (URB597, 10 mg/kg, i.p.), ¶ vs URB-20 (URB597, 20 mg/kg, i.p.) while injection of VDM-11 decreased the contents of 5-HT in a dose-dependent fashion (Panel D; $F_{(4, 45)} = 208.677$; $P < 0.0001$;* vs Control/VEH, α vs VDM-10 (VDM-11, 10 mg/kg, i.p.), € vs VDM-20 (VDM-11, 20 mg/kg, i.p.). All comparisons determined by a one-way ANOVA followed by Scheffé's post hoc where $P < 0.001$ considered statistically different.

rebound period (Fig. 5, Panel D; $F_{(4, 45)} = 208.677$; $P > 0.05$). From this studies, it is implied that URB597 showed more efficiency in promoting enhancement of 5-HT compared to SR141716A whereas VDM-11 caused reversed effects.

In the last study, the AD contents during TSD were no statistical different among the experimental trials (Fig. 6, Panel A; $F_{(4, 45)} = 1.067$; $P > 0.05$). Intriguing results were found when animals received SR141716A after TSD since the CB_1 cannabinoid antagonist increased in a dose-dependent fashion the contents of AD (Fig. 6, Panel B; $F_{(4, 45)} = 6352.478$; $P < 0.0001$). Comparable results were observed when URB597 was injected to rats since this compound caused an increase in a dose-dependent fashion in AD levels after TSD (Fig. 6, Panel C; $F_{(4, 45)} = 559.624$; $P < 0.0001$). Finally, the levels of AD were decreased in a dose-dependent profile after VDM-11 injection (Fig. 6, Panel D; $F_{(4, 45)} = 438.322$; $P < 0.0001$). Based in the data showed, we suggest that URB597 displayed more efficiency in promoting the increase of AD compared to SR141716A during the sleep rebound period. However, differing results were observed after VDM-11 injections. Taken together, our results indicate that the CB_1 cannabinoid receptor, FAAH and AMT might be involved in the control of homeostasis of neurochemicals in response to sleep deprivation.

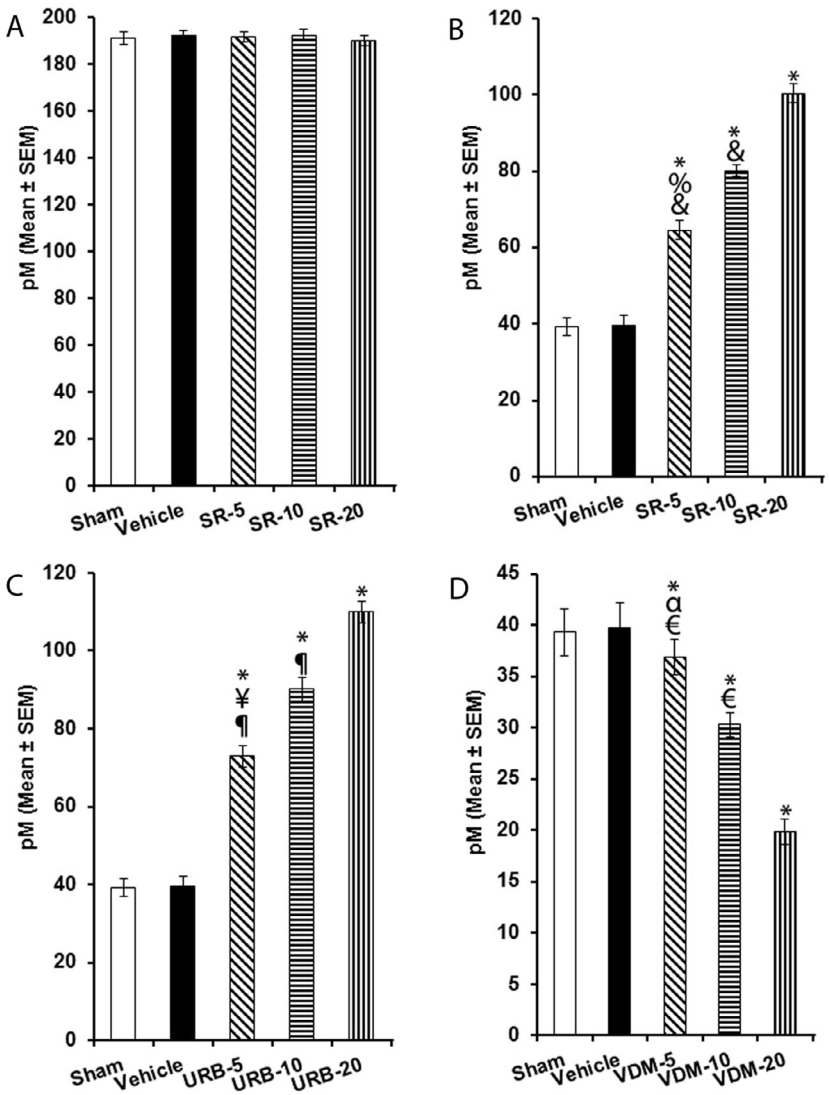

Fig. 6. Effects of SR141716A, URB597 or VDM-11 (10, 20 or 30 mg/kg; i.p. each compound) on extracellular contents of adenosine collected after prolonged waking. Panel A displays that levels of adenosine were increased after sleep deprivation ($F_{(4, 45)} = 1.067$; $P > 0.05$). On the other hand, injection of SR141716A increased in a dose-dependent fashion the levels of adenosine (Panel B; $F_{(4, 45)} = 6352.478$; $P < 0.0001$;* vs Control/VEH, # vs SR-10 (SR141716A, 10 mg/kg, i.p.), & vs SR-20 (SR141716A, 20 mg/kg, i.p.). Moreover, URB597 induced similar effects in adenosine contents after sleep deprivation (Panel C; $F_{(4,4\ 5)} = 559.624$; $P < 0.0001$;* vs Control/VEH, ¥ vs URB-10 (URB597, 10 mg/kg, i.p.), ¶ vs URB-20 (URB597, 20 mg/kg, i.p.). Lastly, injection of VDM-11 diminished the contents of adenosine in a dose-dependent fashion (Panel D; $F_{(4, 45)} = 438.332$; $P < 0.0001$;* vs Control/VEH, α vs VDM-10 (VDM-11, 10 mg/kg, i.p.), € vs VDM-20 (VDM-11, 20 mg/kg, i.p.). All comparisons determined by a one-way ANOVA followed by Scheffé's post hoc where $P < 0.001$ considered statistically different.

To study whether changes in neurochemicals occurred at discrete time points or followed a consistent trend across the sampling period, we analyzed the time course of DA during TSD and the sleep recovery period after injection of SR141716A. As expected, the levels of DA were increased across TSD in experimental groups (Fig. 7, Panel A). However, during sleep rebound period, the extracellular contents of DA were enhanced across 2 h post-administration of SR141716A (Fig. 7, Panel B). Taken together, our results suggest that the CB_1 cannabinoid receptor antagonist might be involved in the modulation of DA homeostasis after sleep deprivation since this compound prolonged the enhancement of DA during sleep recovery period.

DISCUSSION

Although the presumably function of the endocannabinoid system in sleep has been studied, further evidence is needed regarding the role of this complex system in homeostatic regulation of sleep. In this respective, sleep is under control of circadian and homeostatic factors, described in the two-process model of sleep–wake regulation (Franken and Dijk, 2009; Schibler et al., 2015; Borbely et al., 2016). Briefly, the circadian process represents the daily fluctuation of the modulation of sleep threshold levels. Complementary, the homeostatic process symbolizes sleep drive that enhances when waking is maintained beyond habitual bedtime and decreases

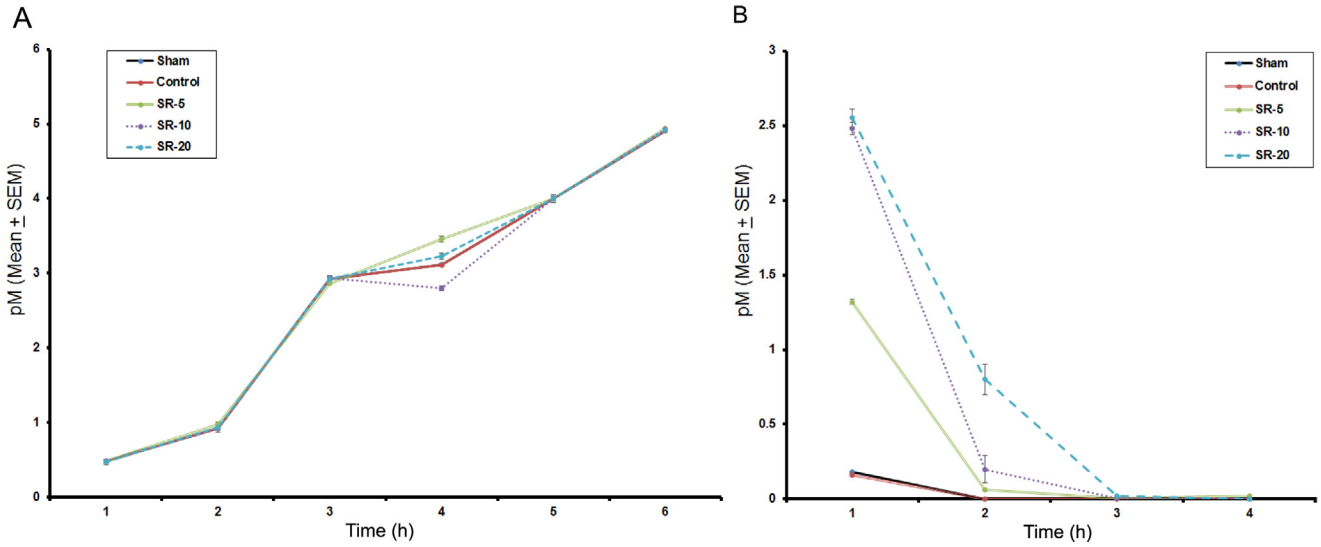

Fig. 7. Endogenous levels of dopamine during the total sleep deprivation (six samples taken at hourly intervals; Panel A) as well as dopamine concentration across the sleep recovery period (4 samples taken at hourly intervals; Panel B). Due to that the experimental trails comprised 11 groups, and to avoid a tightened illustration, for demonstrative purposes in this given example, the graph includes only data of dopamine levels corresponding to the following experimental groups: Sham, Vehicle, SR141716A (5 mg/kg), SR141716A (10 mg/kg), SR141716A (20 mg/kg).

during the next sleep period, which is known as "sleep rebound" (Deboer, 2009; Yasenkov and Deboer, 2012; Curie et al., 2013; Mang and Franken, 2015).

To provide a further insight of the role of the endocannabinoid system in sleep homeostasis modulation, we report the effects of the CB_1 cannabinoid receptor blockade as well as the inhibition of either FAAH or AMT in sleep homeostasis after a short-lasting total sleep deprivation period. The evidence from the present study shows that the CB_1 cannabinoid receptor antagonist, SR141716A, blocked the sleep rebound by inducing alertness in concordance with previous reports. For example, preceding studies have demonstrated that administrations of SR141716A promote waking and diminish SWS and REMS (Santucci et al., 1996; Murillo-Rodríguez et al., 2001, 2003). Furthermore, the CB_1 cannabinoid receptor KO mice show a reduction in SWS during sleep recovery period (Pava et al., 2014; Silvani et al., 2014). However, evidence showing an apparent opposite modulatory role of the CB_1 cannabinoid receptor in sleep modulation has been published as well. In this regard, injection of the antagonist/inverse agonist AM281 of the CB_1 cannabinoid receptor immediately after TSD did not obstructed sleep rebound (Pava et al., 2016). Moreover, SWS was increased during the dark period after administrations of higher dose of CP47497, a cannabinoid receptor agonist, while reduction in this sleep stage was observed across the light period (Pava et al., 2016). The intriguing but as yet unexplained results that CB_1 cannabinoid receptor modulates sleep raises questions about the mechanism of action implied. Nevertheless, evidence regarding the role of the CB_1 cannabinoid receptor in sleep rebound has been addressed by others as well (Navarro et al., 2003; Wang et al., 2011; Silvani et al., 2014). For instance, the expression of the CB_1 cannabinoid receptor in thalamus and brainstem is significantly higher during the sleep rebound period (Wang et al., 2011). Taken together, such findings reveal a striking phenomenon regarding the putative role of the CB_1 cannabinoid receptor in sleep homeostasis control.

Diverse pieces of evidence suggest that the network of the endocannabinoid system, which has been partially studied, might govern the sleep–wake cycle. Along with the CB_1 cannabinoid receptor, the degrading enzyme FAAH for anandamide (Seierstad and Breitenbucher, 2008; Otrubova et al., 2011; Justinova et al., 2015; Wei et al., 2015; Panlilio et al., 2016) also appears to contribute in sleep modulation. Consistent with this idea, expression of FAAH in several brain areas has been described lower after sleep deprivation (Wang et al., 2011). Furthermore, FAAH-KO mice display higher values of SWS (Huitrón-Reséndiz et al., 2004) while the pharmacological inhibition of FAAH by administrations of URB597 in rats promotes waking (Murillo-Rodríguez et al., 2007, 2011). Conversely, it has been shown that administrations of FAAH inhibitor AM3506 promote an increase in SWS (Pava et al., 2016). According to the authors, mice treated with a systemic injection of AM3506 (10 mg/kg) during the dark period showed a significant enhancement in SWS whereas no effects across the light period were observed. Despite the tacit findings in our study, the discrepancies between current results and comparable studies might be addressed by methodological differences. For example, dosage, sleep analysis procedure, species, pharmacological time administration, among other variables might provide an alternative reasonable explanation between opposite results. For instance, Pava et al. (2016) used rotating disk paradigm for sleep deprivation studies whereas in the current report, gentle manipulation was handled. Differences in techniques used for sleep deprivation have provided different outcomes in intensity of sleep rebound (Rechtschaffen et al., 1999; Colavito et al., 2013; Pires et al., 2016). Additionally, the pharmacodynamics between the compounds might exert influence on the

effects reported among comparable reports since AM3506 is more potent than URB597 (Godlewski et al., 2010; Panlilio et al., 2016). In spite of the differences in experimental conditions between tantamount studies, neurobiological basis might contribute as key determinant of sleep control by FAAH since the exact mechanism of action of FAAH in sleep homeostasis is still debated. Nonetheless, the available data suggest that FAAH may be implicated in sleep recovery. Indeed, future work is needed to address an integrated portrait of the neurobiological role of the FAAH in sleep homeostasis.

On the other side, it remains unclear how anandamide is transported across the cell membrane. Though still incomplete, the limited available data suggest that a putative transporter controls the anandamide trafficking (Cravatt et al., 2001; Kathuria et al., 2003; Makriyannis, 2014; Fezza et al., 2014; Batista et al., 2014; Nicolussi and Gertsch, 2015). Regarding the role of AMT in sleep modulation, this issue has been poorly studied. At this date, scanty reports indicate that blocking AMT by VDM-11 at the beginning of the lights-off period diminishes alertness and enhances sleep (Murillo-Rodríguez et al., 2008). In addition, microdialysis perfusion of AMT inhibitor OMDM-2 into a sleep-related brain area such as the paraventricular thalamic nucleus increases sleep whereas diminishes waking as well reduces the extracellular levels of DA (Murillo-Rodríguez et al., 2013). Noteworthy, in the current study when VDM-11 is administered after sleep deprivation, rats displayed sleep rebound by enhancing SWS and REMS. These findings suggest that blocking AMT might increase anandamide signaling and likely VDM-11 would not interfere with the homeostatic regulation of sleep. It is unclear, however, whether AMT is able of evoking control over sleep homeostasis.

In separate studies, we evaluated the impact of the CB_1 cannabinoid receptor blockade or the inhibition of either FAAH or AMT in contents of neurochemicals after prolonged waking. We would like to state that while AcbC is a good target for sampling DA it might not be a good option for NE, EP, 5-HT or AD sampling. However, these neurochemicals have been collected and measured from AcbC as previously reported (Salem and Hope, 1999; Del Arco et al., 2008; Hernandez et al., 2008; Rada et al., 2010; Cannazza et al., 2012; Dobbs and Mark, 2012; Munari et al., 2013; Pajski and Venton, 2013; Dautan et al., 2014). Thus, these evidences indicate the trustworthy of our study. Overall, systemic administration of SR141716A or URB597 before the sleep rebound period enhanced dose-dependently the extracellular levels of DA, NE, EP, 5-HT and AD whereas VDM-11 caused opposite results. Remarkably, the contents of DA were increased 2 h post-SR141716A injection during sleep recovery period. How can we interpret this data? Despite that we did not develop mechanicist experiments, it is possible to draw the following hypothesis: An interacting cross-talking between the endocannabinoid and wake-related systems might be involved in described effects. This statement is based in the subsequent evidence: First, it has been described the presence of the CB_1 cannabinoid receptor in wake-related brain areas including hypothalamus and pons (Miederer et al., 2013; Soni et al., 2014). Second, there is an interactive relationship between the CB_1 cannabinoid receptor and DA system (Degroot et al., 2006; Davis and Nomikos, 2008; Martin et al., 2008; Kleijn et al., 2012; Dazzi et al., 2014). Third, previous reports have shown that SR141716A enhances NE contents (Tzavara et al., 2001, 2003; Bedse et al., 2015). Fourth, there is evidence regarding the interaction between the CB_1 cannabinoid receptor and AD system (Murillo-Rodríguez et al., 2003; Cerri et al., 2014) suggesting that SR141716A would be blocking the CB_1 cannabinoid receptors present in cholinergic neurons which are placed in basal forebrain, and they are presumably responsible for AD release (Blanco-Centurion et al., 2006; Huang et al., 2011).Taken together, it might be reasonable to consider that SR141716A or URB597 might activate the wake-related neurotransmission systems and control sleep homeostasis. Nevertheless, further studies are needed to clarify whether the endocannabinoid system interacts with wake-related components to control sleep homeostasis.

An alternative explanation implies that FAAH inhibition might promote an endogenous accumulation of monoamines as suggested by others (Solinas et al., 2006; Murillo-Rodríguez et al., 2007, 2011; Cassano et al., 2011; Bambico et al., 2016) or involving the activity of the PPARα receptors as indicated (Melis et al., 2008; Luchicchi et al., 2010; Bedse et al., 2015). Thus, it is likely to speculate that the increase in contents of monoamines or AD caused by URB597 during sleep rebound might be via the engaging of PPARα receptors. To support this idea, we have described that injection of Wy14643 (PPARα agonist) promoted W whereas decreased sleep. Besides, this compound increased monoamines as well as AD levels (Mijangos-Moreno et al., 2016). To strengthen this alternative explanation, additional evidence indicates that FAAH inhibition enhances contents of oleoylethanolamide over anandamide levels (Fegley et al., 2005). Importantly, oleoylethanolamide is the endogenous ligand for PPARα receptors (Fu et al., 2005; Campolongo et al., 2009; Bilbao et al., 2015) and it has been demonstrated that injections of oleoylethanolamide promote waking (Murillo-Rodríguez et al., 2007, 2011). Since PPARs are found to be involved in energy homeostasis (Tovar-Palacio et al., 2012; Tain et al., 2015) they might be engaged in sleep homeostasis. Therefore, URB597 may contribute to modulate sleep homeostasis via activation of PPARα receptors by the endogenous accumulation of oleoylethanolamide. Despite this tempting scenario, no further evidence is available regarding the possible link between FAAH and PPARα receptors in sleep homeostasis.

Lastly, the injections of VDM-11 allowed the sleep rebound in rats subjected to prolonged waking schedule. Moreover, it is our understanding that the only available data regarding the effects of AMT inhibition in sleep-related neurochemicals demonstrate that VDM-11 decreases DA levels as measured by HPLC means (Murillo-Rodríguez et al., 2013). The results found in the current study lead us to the following critical question: What is the mechanism activated by VDM-11 that allowed the sleep recovery in sleep-deprived rats? We cannot

answer this question yet, but it might be likely that VDM-11 induces sleep via anandamide since it has been described that VDM-11 promotes the endogenous accumulation of this endocannabinoid (de Lago et al., 2004, 2005). In line with this idea, several pieces of evidence supports that anandamide promotes sleep (Mechoulam et al., 1997; Murillo-Rodríguez et al., 1998, 2001, 2003, 2011; Herrera-Solis et al., 2010; Rueda-Orozco et al., 2010). Taken together, our data suggest that blocking AMT does not interfere with sleep rebound. Nonetheless, we cannot rule out the possibility that the effects described in our study might be mediated by concomitant unknown mechanisms.

CONCLUSION

Our results fortifies the body of literature indicating that administrations of either SR141716A or URB597 modulate sleep. Precisely, injections of both compounds promoted wake-inducing effects during sleep recovery period whereas injections of VDM-11 induced sleep across sleep recovery period. The present findings remark that the animals treated with SR141716A or URB597, increased alertness during sleep rebound likely due to an endogenous accumulation of DA, NE, EP, 5-HT or AD. However, VDM-11 allowed the display of the sleep rebound in sleep-deprived rats by likely decreasing the extracellular contents of wake-related neurochemicals. Thus, for all data combined, the presumably role of the endocannabinoid system in the modulation of the sleep homeostasis after sleep deprivation is highlighted. To add pieces to the puzzle of the putative mechanism of action of SR141716A, URB597 and VDM-11 in sleep homeostasis regulation, it is worthy to note that the endocannabinoid system has been described in astrocytes (Stella, 2010; Bosier et al., 2013; Kőszeghy et al., 2015; Oliveira da Cruz et al., 2016). Astrocytes have an important role in sleep homeostasis (Frank, 2011, 2013) with relevant functions by releasing AD as gliotransmitter (Schmitt et al., 2012; Nadjar et al., 2013; Hines and Haydon, 2014; O'Donnell et al., 2015). Thus, taking into account all these findings, CB_1 cannabinoid receptor, FAAH or VDM-11 located in astrocytes in sleep-related structures (Kőszeghy et al., 2015; Kovács et al., 2016) might be playing an undescribed role in sleep homeostasis. Indeed, the interactions between the CB_1 cannabinoid receptor, FAAH and AMT placed in neurons and astrocytes during sleep homeostasis remain to be described. Further investigations regarding this issue would be basis of clinical research aimed to explore the pharmacological manipulation of the endocannabinoid system with potential therapeutical benefits in sleep homeostatic disorders.

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