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Chemoenzymatic synthesis and cannabinoid activity of a new diazabicyclic amide of phenylacetylricinoleic acid

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ABSTRACT

Endocannabinoids (eCBs) are endogenous neuromodulators of synaptic transmission. Their dysfunction may cause debilitating disorders of diverse clinical manifestation. For example, drug addiction, lack of sex desire, eating disorders, such as anorexia or bulimia and dyssomnias. eCBs also participate in the regulation of core temperature and pain perception. In this context, it is important to recognize the utility of cannabinoid receptor **1** (CB1R) agonists, natural as Δ^9-tetrahydrocannabinol (THC) or synthetic as Nabilone as useful drugs to alleviate this kind of patients' suffering. Therefore, we have developed a new drug, (*R*,*Z*)-18-((1*S*,4*S*)-5-methyl-2,5-diazabicyclo[2.2.1]heptan-2-yl)-18-oxooctadec-9-en-7-yl phenylacetate (PhAR-DBH-Me), that appears to bind and activate the CB1R. This diazabicyclic amide was synthesized from phenylacetylricinoleic acid and (1*S*,4*S*)-2,5-diazabicyclo[2.2.1]heptane. To test its cannabinergic properties we evaluated its effects on core temperature, pain perception, and the sleep-waking cycle of rats. Results indicate that 20 and 40 mg/kg of PhAR-DBH-Me readily reduced core temperature and increased pain perception threshold. In addition, 20 mg/kg increased REM sleep in otherwise normal rats. All these effects were prevented or attenuated by AM251, a CB1R antagonist. Place preference conditioning studies indicated that this molecule does not produce rewarding effects. These results strongly support that PhAR-DBH-Me possesses cannabinoid activity without the reinforcement effects.

Endocannabinoids (eCBs) are molecules synthesized by neurons of all mammalians and insects known.[1] eCBS modulate the synaptic transmission participating in the generation of several types of behavior.[2] Therefore, their dysfunction may cause debilitating disorders of diverse clinical manifestation. For example, drug addiction,[3] lack of sex desire and eating disorders,[4] such as anorexia or bulimia[5] and dyssomnias.[6] Additionally, the activation of the cannabinoid receptor 1 (CB1R) modulates pain perception and core temperature.[7] Mounting evidence indicates that in schizophrenia eCBs may be dysfunctional.[8]

Marijuana derivatives, such as Δ^9-tetrahydrocannabinol (THC, Fig. 1) commercially available as Dronabinol® or THC plus cannabidiol (Sativex®) and several synthetic compounds as Nabilone (Cesamet®), among others, produce several therapeutic effects.[9,10]

For example, they are appetite stimulants in patients suffering from wearing-off caused by acquired immunodeficiency syndrome

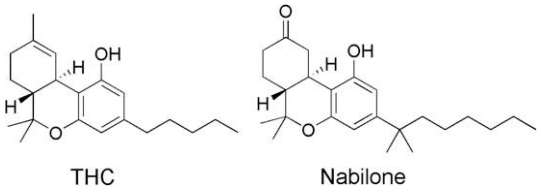
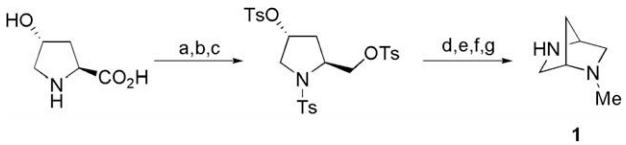


Figure 1. Chemical structures of THC and Nabilone.



Scheme 1. Chemical synthesis of (1*S*,4*S*)-2-methyl-2,5-diazabicyclo[2.2.1]heptane (**1**) from *trans*-4-hydroxy-(*S*)-proline. Reagents and conditions: (a) TsCl, Na_2CO_3, H_2O, 94%; (b) $NaBH_4$, $BF_3{\cdot}Et_2O$, THF, 85%; (c) TsCl, C_5H_5N, toluene, 20 h, 83%; (d) $PhCH_2NH_2$, toluene, reflux, 96%; (e) HBr 40%, 96%; (f) HCO_2H, HCOH, MeOH, reflux, 73%; (g) H_2, Pd/C, MeOH 80%.

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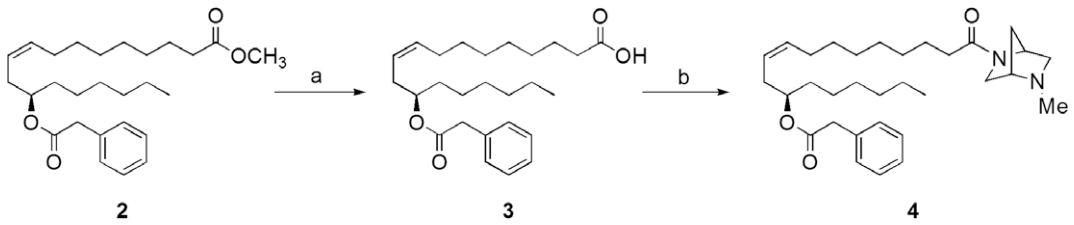
or chemotherapy. Cannabinoids can also be used as antiemetic drugs in patients under chemotherapy.[11] Pain is another indication for this class of compounds.[9,12] There is information indicating its successful use to control glaucoma.[13] Likewise, there is mounting experimental evidence indicating that cannabinoids may be useful to control epilepsy,[14] anxiety,[15] and insomnia.[6] Despite these advances, the toxic or lethal effects caused by cannabinoids have not been discussed thus far.[16] In addition, blocking of the CB1R receptor by drugs such as SR141716a, may induce side effects that compromise patients' health or life, as has been suggested by the experience gained with the use of SR141716a for food intake control in obese patients.[17] Therefore, it is important to recognize the



Scheme 2. Chemoenzymatic synthesis of compound **4**. Reagents and conditions: (a) Novozym 435, water/acetone 6:4, phosphate buffer 100 mM pH 7.5, 37 °C, 1 h, quantitative yield; (b) (1) PvCl, CH_2Cl_2; (2) diamine **1**, 80%.

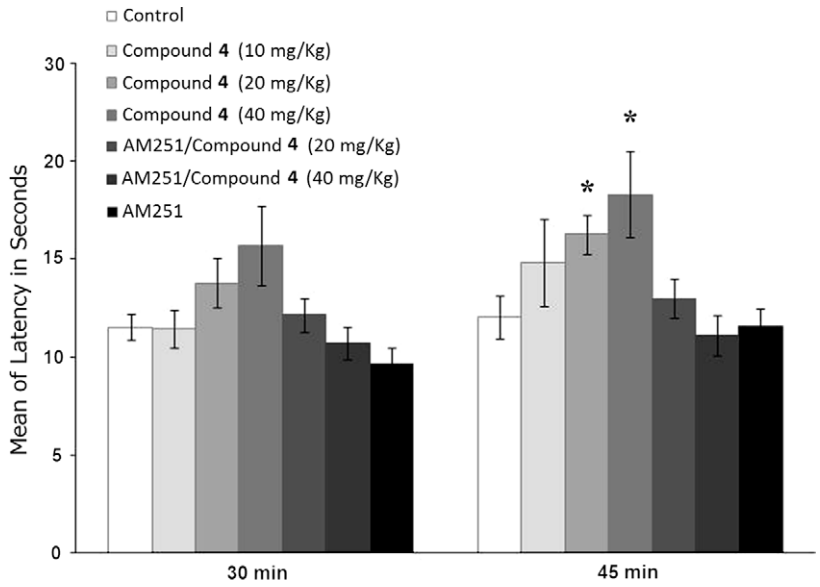


Figure 2. This figure depicts the effect of compound **4** at three doses on pain perception as evaluated with the hot-plate paradigm. Such an effect has been blocked by AM251. p <0.05, compared to control.

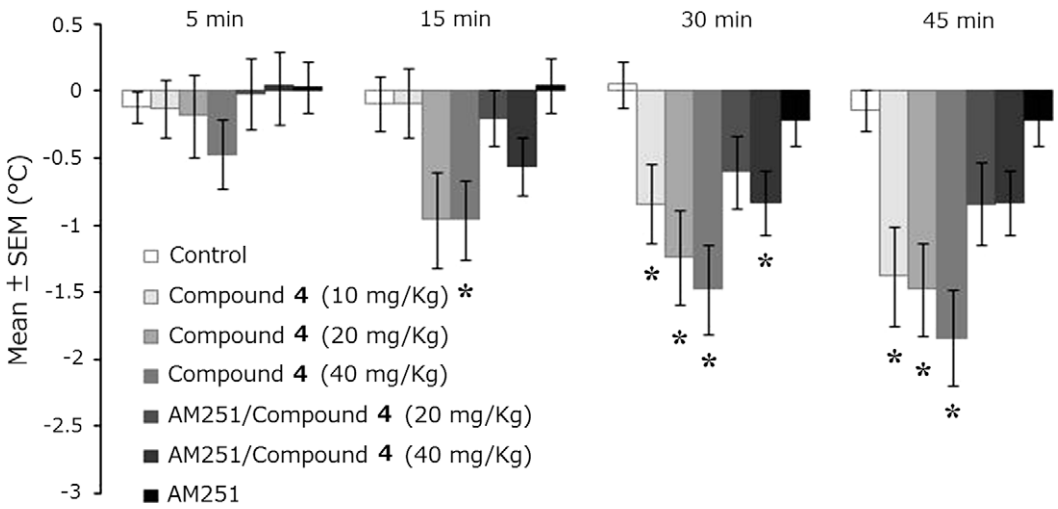


Figure 3. This figure illustrates the effect of three doses of compound **4** on the core temperature. There is a reduction of about 1 °C in 15 min after the ip injection. This effect remains for 30 more minutes, and is hampered by AM251. p <0.05 compared to control.

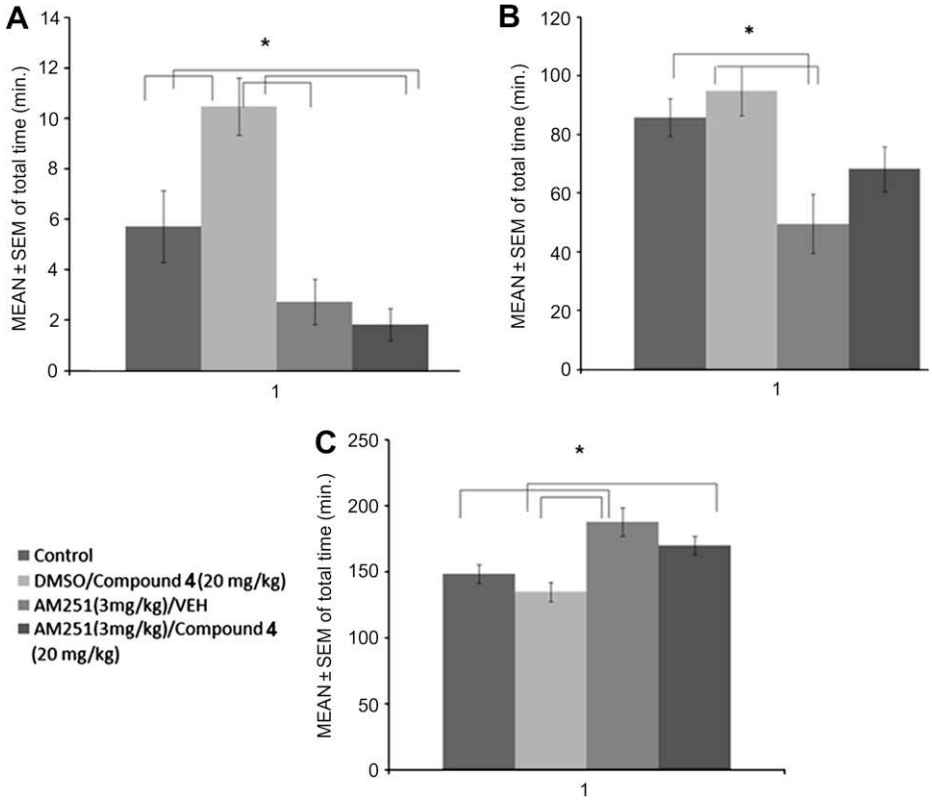


Figure 4. These graphs illustrate the effect of compound **4** on the sleep-waking cycle of rats during 4 h. In A the effect on REM sleep is depicted. Likewise for NonREM sleep in B and for waking in C. Observe the important REM sleep inducing effect caused by compound **6** compared to control. This effect is blockaded with AM251, a selective CB1 antagonist. p <0.05.

therapeutic potential of cannabinoids and develop new drugs with reduced side effects, and with potential clinical applications in the treatment of several diseases.

We have recently synthesized a new diazabicyclic amide derivative of phenylacetylrincinoleic acid (*R*,*Z*)-18-((1*S*,4*S*)-5-methyl-2,5-diazabicyclo[2.2.1]heptan-2-yl)-18-oxooctadec-9-en-7-yl phenylacetate (PhAR-DBH-Me, **4**), and tested its effects on some physiological variables, revealing that it possesses cannabinoid activity and that such an effect is mediated by the CB1R. This new compound **4** has been prepared starting from *trans*-4-hydroxy-(*S*)-proline and castor oil, both compounds acting as natural sources of chirality. In the first step, (1*S*,4*S*)-2-methyl-2,5-diazabicyclo[2.2.1]heptane (**1**) was synthesized according to the procedure recently described by Regla, Juaristi and co-workers (Scheme 1).[18]

The synthetic strategy for the preparation of **4** is outlined in Scheme 2. The preparation of methyl 12-phenylacetylricinoleate (**2**) took place with the recently reported conditions.[19] A key step in the synthetic methodology was the development of a new strategy for the regioselective chemoenzymatic hydrolysis of **4** to obtain 12-phenylacetylricinoleic acid (**3**), a described precursor for the synthesis of 12-acyl substituted ricinoleylamides.[20,21] Compound **4** was obtained in 70% yield by the mixed anhydride method. Thus, treatment of **3** with pivaloyl chloride in a mixture of Et_3N and dichloromethane at 0 °C for 15 minutes to produce the corresponding mixed anhydride was followed by reaction with diamine **1**.[22]

Our physiological and behavioral[22] results indicate that three doses of PhAR-DBH-Me (**4**) reduced core temperature by about 1.5–2.0 °C; such doses also increased rats' latency to exhibit signs of discomfort in the hot-plate test. Moreover, AM251 successfully blocked these effects; however, AM251 did not cause any effect by itself at the dose levels tested. These results suggest that PhAR-DBH-Me (**4**) is a CB1R agonist (see Figs. 2 and 3).

Regarding the sleep-waking cycle, results indicate that 20 mg/kg of **4** increased REM (rapid eye movement) sleep total time during 4 h, with a simultaneous tendency to increase NREMS total time. A tendency to reduce waking-up was also documented. These changes in REM sleep were blocked by AM251 (see Fig. 4). These PhAR-DBH-Me-induced changes have been observed with agonists and antagonists of the CB1R receptor.

Finally, PhAR-DBH-Me (**4**) was tested at the dose of 20 mg/kg to evaluate its capacity to induce addiction. A conditioned place preference paradigm was utilized to determine if **4** is capable of inducing signs of dependence. Data indicated that **4** failed to induce signs of dependence (see Fig. 5).

In conclusion, PhAR-DBH-Me (**4**) is able to induce effects similar to those observed with well-known cannabinoids. Moreover, such effects are prevented by the administration of the CB1R antagonist

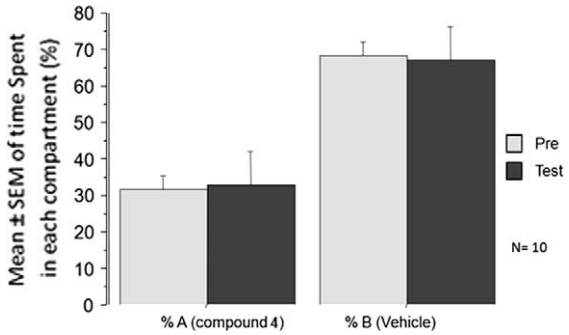


Figure 5. This figure illustrates the absence of rewarding effect of compound **4**. As it can be seen, compound **4** does not modify the time spend in the compartment A (associated to the administration of this drug).

AM251. Further experiments will clarify if this drug induces adverse side effects.

Acknowledgements

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22. *General*: ^{1}H and ^{13}C NMR spectroscopy was carried out on a JEOL Eclipse instrument at 200 and 300 MHz, with TMS as an internal standard and $CDCl_3$ as solvent. The IR spectra were carried out on a Bruker spectrophotometer Tensor 27. The $[\alpha]_D^{20}$ values were determined on a 341 Perkin–Elmer polarimeter, at 1 dm cell length. HRMS was determined on a JEOL JMS-SX102A instrument. Silica gel chromatography: 70–230 mesh. Multiplicity keys: s = singlet, d = doublet, t = triplet, c = quartet, q = quintet, m = multiplet, br = broad, dd = doublet of doublets. *Typical procedure for the enzymatic hydrolysis for the preparation of phenylacetylricinoleic acid* (**3**). To a solution of methyl 12-phenylacetylricinoleate (7 g, 16.25 mmol) in acetone (360 mL), were added water (120 mL), phosphate buffer 100 mM pH 7.5 (240 mL) and Novozym 435 (3.6 g (10 mg/mL). The reaction mixture was incubated for 1 h at 37 °C and 250 rpm, monitoring the reaction by TLC (hexane/EtOAc 9:1). The mixture was filtered through Celite and concentrated under reduced pressure in order to remove acetone. The aqueous layer was extracted with dichloromethane (3 × 50 mL), the organic layer was dried with anhydrous Na_2SO_4 and concentrated under reduce pressure to yield 6.7 g of pure **3** as a colorless oil in 99% yield. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.86 (t, *J* = 6.2, 3H), 1.2–1.35 (m, 16H), 1.49 (m, 2H), 1.59–1.66 (m, 2H), 1.96–1.99 (m, 2H), 2.24–2.27 (m, 2H), 2.34 (t, *J* = 7.4 Hz, 2H), 3.59 (s, 2H), 4.87 (q, *J* = 6.2 Hz, 1H), 5.21–5.33 (m, 1H), 5.37–5.50 (m, 1H), 7.23–7.32 (m, 6H), 9.44 (br s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.0, 22.4, 24.6, 25.1, 27.2, 28.9, 29.0, 29.1, 29.4, 31.6, 31.8, 33.5, 33.9, 41.7, 74.4, 124.1, 126.9, 128.4, 129.1, 132.5, 134.2, 171.3, 179.9. *(R,Z)-18-((1S,4S)-5-methyl-2,5-diazabicyclo[2.2.1]heptan-2-yl)-18-oxooctadec-9-en-7-yl phenylacetate* (**4**, PhAR-DBH-Me): In a three-necked round-bottom flask, 2 g, (4.8 mmol) of **3** and 667 μL (4.8 mmol) of Et_3N were dissolved in CH_2Cl_2 (25 mL). The reaction mixture was cooled to 0 °C in an ice bath and pivaloyl chloride (591 μL, 4.8 mmol) was added and stirred at the same temperature for 15 min. Afterwards, (1*S*,4*S*)-2-methyl-2,5-diazabicyclo[2.2.1]heptane (537 mg, 4.8 mmol) was added and stirring continued for 15 min at 0 °C. The organic layer was washed with water (3 × 10 mL), dried with anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain a liquid, which after chromatographic purification (CH_2Cl_2/MeOH 95:5) afforded 1.76 g (74%) of PhAR-DBH-Me (**4**) as a colorless oil. ^{1}H NMR (300 MHz, $C_2D_2Cl_4$) −130 °C: δ = 0.90 (t, *J* = 7.2 Hz, 3H), 1.28–1.35 (m, 16 H), 1.58–1.66 (m, 4 H), 1.77 (d, *J* = 10.5, 1H), 2.02 (c *J* = 6.9, 2H), 2.11–2.24 (m, 2H), 2.19 (t, *J* = 7.8 Hz, 2H), 2.57 (s, 3H), 2.91–3.03 (m, 2H), 3.31 (d, *J* = 10.8 Hz, 2H), 3.58 (s, 2H), 3.71 (br s, 2H), 3.82 (d, *J* = 12, 2H), 4.88 (q, *J* = 6.6 Hz, 1H), 5.31–5.34 (m, 1H), 5.44–5.48 (m, 1H), 7.26–7.29 (m, 5H). $[\alpha]_D^{20}$ = −4.2 (*c* 1, MeOH). IR ν_{max} (film) cm^{-1}: 3441, 3062, 2928, 2855, 1731, 1644, 1430, 1255, 1161, 1028, 763, 723. MS (EI) *m*/*z* (%) = 511 (100) $[M^+]$, 375 (64), 113 (18), 82 (55), 68 (9). HRMS-FAB$^+$: *m*/*z* calcd for $C_{32}H_{50}N_2O_3$ $[M+H]^+$: 511.3900; found: 511.3888.
Experimental methods for the physiological and behavioral evaluations: *Subjects*: Adult Wistar rats, weighting 250–350 g were used in this study. All animals were housed individually in Plexiglas cages. They were maintained at an ambient temperature of 22 ± 1 °C and a controlled 12:12 h light–dark cycle (08:00 am–08.00 pm; lights off at 08:00 am) throughout the study. Food and water were available ad libitum. Animals were treated according to the Norma Oficial Mexicana (NOM-062-ZOO-199), the Guide for Care and Use of Laboratory Animals established by the National Institutes of Health, and the European Community Council Directive 86/609/ EEC. Additionally, our protocol was approved by the Research and Ethics Committee of the Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM). Every effort was made to minimize the number of animals used and their potential suffering. *Effects on core temperature and pain perception*: Seventy rats were used in this part of the study. They were randomly assigned to seven groups to determine a PhAR-DBH-Me (**4**) dose–response curve (0, 10, 20 and 40 mg/kg ip). Two additional groups (*n* = 10, each group) received 20 or 40 mg/kg of PhAR-DBH-Me (**4**) 15 min after the administration of AM251 (3 mg/kg, ip), a selective CB1 receptor (CB1R) antagonist to block the effect. One last group was included to test the effect of the AM251 alone. Core temperature was measured by using a rectal probe and pain perception by using the hot plate method. *Sleep-waking cycle recordings*: (a) *Surgery*: Rats (*n* = 6 per group) were stereotaxically implanted under anesthesia (cocktail: 66 mg/kg ketamine, 0.26 mg/kg xylazine, and 1.3 mg/kg acepromazine) with two electrodes inserted into the hippocampus (*P* = 4.0, *L* = 2.5, *V* = 2.5) according to the Paxinos and Watson atlas[23] for electroencephalographic (EEG) recordings. Two additional screw electrodes were implanted into the frontal bones for grounding the animal. Two twisted wire electrodes were placed into the neck musculature for electromyographic (EMG) recordings. (b) *Sleep recordings*: After surgery, animals were monitored and provided with the proper veterinary aid to speed their recovery up. They were allowed to recover for 10 days. Upon the completion of this period, rats were habituated to the recording conditions for 24 h. Once the habituation period was completed, rats were divided into different experimental groups. Vehicle group (control) was administered with two ip injections. First injection was DMSO (1 ml/kg), the vehicle for AM251 and 15 min later vegetal oil (1 ml/kg), the vehicle for PhAR-DBH-Me (**4**). PhAR-DBH-Me group was injected with DMSO and 15 min later with PhAR-DBH-Me (**4**). Group AM251, was injected with this drug and 15 min later injected with vegetal oil. Finally, the AM251 + PhAR-DBH-Me group, was injected with these two drugs 15 min apart and in this sequence. All injections were performed at the beginning of the dark phase of the light-dark cycle. Immediately after the second administration, the sleep-waking cycle was recorded for 12 h. The EEG and EMG signals were amplified with a Grass Model 7 polygraph, Amplifier Model 7P511, in a frequency range of 1–30 Hz, and 30–100 Hz, respectively. Signals were acquired and analyzed with the ICELUS® software. *Data analysis*: Polygraphic recordings were analyzed every 12 s and classified according to the following vigilance stages: wakefulness (W), slow wave sleep (SWS), and rapid eye movement sleep (REMS). Electrophysiological criteria were used to define these stages of vigilance as follows: W was characterized by the EEG expressing mixed low fast voltage and theta activity, as well as high muscle activity. In SWS, the EEG showed delta waves and decreased EMG amplitude. Finally, in REMS the EEG expressed theta activity without EMG activity (postural atonia). The time spent in W, SWS, and REMS per hour was calculated during 12 h period. SWS and REM sleep latency was also calculated by measuring the time elapsed from the start of the sleep recording to the first SWS bout. REMS latency was considered from the first SWS bout to the first REMS bout. Frequency and average duration of REMS bouts were also calculated. *Statistics*: Results of REMS, SWS, and W were compared by a mixed analysis of variance (ANOVA) with a Greenhouse-Geisser correction, and subsidiary ANOVAs to detect changes per hour with an LSD post hoc test used only for specific comparison when indicated by mixed ANOVA. Sleep latencies and average duration of REMS episodes were analyzed with one way ANOVA and post hoc LSD. Finally, frequency of REMS was analyzed with Kruskal–Wallis test with post hoc U-Mann-Whitney and Bonferroni correction.
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