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Oleamide restores sleep in adult rats that were subjected to maternal separation

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ABSTRACT

Maternal separation (MS) induces a series of changes in rats' behavior; among them a reduction in spontaneous sleep. One potentially impaired system is the endocannabinoid system (eCBs), since it contributes to generate sleep. To investigate if there are situations early in life that affect the eCBs, which would contribute to make rats vulnerable to suffering insomnia, we studied the rodent model of MS. Rats were separated from their mothers for 3 h-periods daily, from postnatal day (PND) 2 to PND 16. Once they gained 250 g of body weight (adult rats), they were implanted with electrodes to record the sleep–waking cycle (SWC). MS rats and non-MS (NMS) siblings were assigned to one of the following groups: vehicle, oleamide (OLE, an agonist of the cannabinoid receptor 1, CB1R), OLE + AM251 (an antagonist of the CB1R) and AM251 alone. Expression of the CBR1 receptor was also analyzed in the frontal cortex (FCx) and in the hippocampus (HIP) of both NMS and MS rats. Results indicated that MS induced a reduction in both non-rapid eye movement (NREM) and rapid eye movement (REM) sleep with the consequent increase in waking (W) as compared to NMS siblings. OLE normalized the SWC, and AM251 blocked such an effect. CB1R expression was reduced in the FCx and in the HIP of MS rats. Our results indicate that MS reduces sleep and CB1R expression and OLE improves sleep in adult rats.

1. Introduction

The international classification of sleep disorders (American Academy of Sleep Medicine, 2005) considers there are about 85 sleep disorders, among them 11 are insomnia type. Among the latter, the psychophysiological and idiopathic insomnia are the most insidious. To date, we do not know what pathophysiological changes underlie this type of insomnia. Everyday life stressors trigger and maintain these conditions. However, in most cases, stressors are of the same kind that the great majority of the population deals with without slipping into insomnia. In this context, it seems like patients suffering insomnia may have some predisposition that makes them vulnerable to this disease. Given this situation, the model of maternal separation (MS) in rats seems to offer a model to study this type of insomnia (Feng et al., 2011).

Maternal care (MC) is a very important stimulant the newborn receives early in life (Meaney and Szyf, 2005). In rats, MC consists of nursing the pups, licking, and looking after their needs. Because not all rat mothers provide the same MC, in terms of frequency, some of them receive low MC (Liu et al., 2000a). It has been shown that rats that were provided high MC when they were pups are much more successful in performing tasks such as solving mazes when they become adults, than those that received low MC (Liu et al., 2000b). Moreover, the high MC rats have more neurons in the hippocampus (Bredy et al., 2003), more expression of the subunit NR2A of the NMDA receptor, more acetylcholine, more brain derived neurotrophic factor (BDNF) and other molecules that seem to be important for memory processes than the rats that were raised with low MC (Liu et al., 2000b). In addition, rats that were subjected to a more aggressive procedure, i.e., rats separated from their mothers (MS) for 6 h every day for 10 days, have more expression of orexin A and corticotropin releasing factor (CRH) in the hypothalamus, more orexin receptor 1 in the FCx and more orexin receptor 2 in the HIP than NMS rats (Feng et al., 2007). The MS rats have also a smaller pineal gland and less melatonin in the pineal gland and hypothalamus than NMS rats (Feng et al., 2011). All these facts contribute to enhance W.

Regarding the behavior, MS rats exhibit signs of depression, according to the forced swimming test (Porsolt et al., 1977; Lambas-Senas et al., 2009). A review of the literature on the sleep of MS rats shows this topic to be controversial. Some studies indicate

Abbreviations: eCB, endocannabinoids; eCBs, endocannabinoid system; MS, maternal separation; NMS, non-maternal separation; PND, postnatal day; SWC, sleep waking cycle; OLE, oleamide; CB1R, cannabinoid receptor 1; NREM, non-rapid eye movement sleep; REM, rapid eye movement sleep; W, waking; FCx, frontal cortex; HIP, hippocampus.

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that MS female rats show an increase in REM sleep when adults (Tiba et al., 2008); while MS male rats show a decrease, but only during the light phase (Feng et al., 2011).

On the other hand, there is evidence indicating that endocannabinoids (eCB) induce sleep, when injected systemically (Cravatt et al., 1995; Mendelson and Basile, 2001), into the ventricle (Herrera-Solis et al., 2010), into the hippocampus (Rueda-Orozco et al., 2010), into the brainstem (Murillo-Rodriguez et al., 2001) or into the preoptic area (Mendelson and Basile, 2001). Likewise, several studies indicate that eCB reduce the level of alertness, and anxiety (Finn, 2010).

Given this context, we investigated whether MS effectively changes the sleep–waking cycle and if such changes are related to a reorganization of the CB1R expression. In addition, oleamide was tested to restore the SWC.

2. Materials and method

2.1. Subjects

Male Wistar rat pups were separated from their mother (MS) from postnatal day 2 (PND2) to PND 16, for a 3 h-period daily (from 11 to 14 h), while another group of pups, composed of the siblings of the MS rats, stayed with the mother at all times (control group, NMS). Once this period was completed, pups subjected to MS were also allowed to stay with their mother until PND22. On PND23, pups of both groups (MS and NMS) were separated permanently from their mother and they were caged in groups of 5 until they gained 250 g of body weight (when rats became adults, approximately at the third month rats reached this body weight). They were maintained at an ambient temperature of 22 ± 1 °C and a controlled 12:12-h light–dark cycle (lights on: 08:00 AM–08.00 PM) throughout the study. Food and water were available ad libitum.

2.2. Surgery

Once rats gained the abovementioned weight (adult rats), they 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 HIP (P = 4.0, L = 2.5, V = 2.5) according to the Paxinos and Watson atlas (1986) to record electroencephalographic (EEG) signals from this structure to determine 3 vigilance states: waking (W), NREM sleep and REM sleep. The theta rhythm is easily recorded from the HIP, helping us to differentiate between W and REM sleep (Prospéro-García et al., 1993; Herrera-Solis et al., 2010). Two additional screw electrodes were implanted into the frontal bones to ground the animal. Two twisted wire electrodes were placed into the neck musculature for electromyographic (EMG) recordings. Animals were treated according to the *Norma Oficial Mexicana* (NOM-062-ZOO-199), the Guide for Care and Use of Laboratory Animals established by the National Institutes of Health, and the European Community Council, Directive 86/609/EEC. Additionally, our protocol was approved by the Research and Ethics Committee of the *Facultad de Medicina*, *Universidad Nacional Autónoma de México* (*UNAM*). Every effort was made to minimize the number of animals used and their potential suffering.

2.3. Chemicals

Oleamide (OLE), dimethylsulfoxide (DMSO) and phenylmethylsulfonylfluoride (PMSF) were all from Sigma (Sigma-Aldrich, Mexico). The CB1R inverse agonist, AM251, was obtained from Cayman Chemical. We decided to use AM251 over SR141716A, another CB1R inverse agonist, for two reasons: 1) it is more specific for CB1R and 2) it is commercially available. OLE and AM251 were dissolved in a mixture of 30% dimethyl sulfoxide (DMSO) in saline. Doses of drugs and volumes will be specified later in the description of each experiment. Tris (hydroxymethyl)-aminomethane, glycine, sodium dodecyl sulfate (SDS), and nonfat dry milk, were all from Bio-Rad. Protease Cocktail Inhibitor Complete Mini was from Roche Diagnostics. All buffers and dissolutions were filtered through 0.22 μm filter units.

2.4. Membrane protein extraction from rat brain

One additional MS and one NMS group of rats (10 rats each group) were sacrificed by neck dislocation and the brain was quickly extracted. The brain sections, FCx and HIP, were homogenized (0.1 g tissue/1 ml) in a buffer solution containing 250 mM sucrose, 1 mM EDTA, 10 mM Tris·HCl buffer, pH 7.6, 10 mM PMSF and a protease cocktail inhibitor complete mini 1 tablet/50 ml (Roche Diagnostics, Rotkreuz, Switzerland). Large tissue debris and nuclear fragments were removed by two low-speed spins (1000 *g*, 10 min, 4 °C). The supernatant (crude membranes) was collected and centrifuged (17,000 *g*, 20 min, 4 °C). The pellet enriched in plasma membranes was re-suspended in buffer solution (85 μl). The protein concentration was measured by means of the Bio-Rad D C protein assay (Bio-Rad, Hercules, CA).

2.4.1. Immunoblotting

Plasma membrane samples (20 μg) were electrophoretically separated in 8% SDS-PAGE and electroblotted to a PVDF membrane (Millipore). Nonfat dry milk (5%, Bio-Rad) in TBS-T (20 mM Tris·HCl, 136 mM NaCl, 0.1% Tween 20, pH 7.6) was added for 1 h. The PVDF membrane was incubated overnight with rabbit polyclonal antibodies against CB1R (1:500, Cayman Chemical), diluted in TBS-T at 4 °C. The membrane was rinsed three times (10 min each) with TBS-T and incubated with the secondary antibody, donkey anti-rabbit IgG coupled to horseradish peroxidase (1:10,000; Santa Cruz) in TBS-T for 1 h at room temperature. Immunoblots were detected using ECL plus detection reagents (Amersham Biosciences) and exposed to autoradiographic film for 1 min (Kodak).

2.5. Sleep recording

After surgery, animals were monitored and allowed to recover for 10 days. Upon 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: OLE (1 mg/kg), AM251 (1.6 mg/kg, equimolar to OLE dose) or a cocktail of both (AM251 + OLE). DMSO (30% in saline) was the vehicle. All treatments were systemically (intraperitoneal) administered 30 min before the lights went on (08.00 am). The groups were formed by: NMS with vehicle (VEH-NMS; n = 10), NMS rats with oleamide (OLE-NMS; n = 10), NMS rats with AM-251 (AM-NMS; n = 10), NMS rats with cocktail (CKT-NMS; n = 10), MS rats with vehicle (VEH-MS; n = 10), MS rats with OLE (OLE-MS; n = 10), MS rats with AM-251 (AM-MS; n = 10), and MS rats with cocktail (CKT-MS; n = 10).

Rats were recorded for 24 h. Polysomnographic recordings started right after the drug administration. EEG and EMG signals were amplified with a Grass Model 7 polygraph, Amplifier Model 7P511, in a frequency range of 1 to 30 and 30 to 100 Hz, respectively. Signals were acquired and analyzed with ICELUS® software (Mark Opp, Ann Arbor, MI, USA).

2.6. Data analysis

Polygraphic recordings were analyzed every 10 s and classified according to the abovementioned vigilance stages: W, NREM, and REM sleep. Electrophysiological criteria were used to define these stages of vigilance as follows: W was characterized by the EEG