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2-AG into the lateral hypothalamus increases REM sleep and cFos expression in melanin concentrating hormone neurons in rats

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ABSTRACT

Orexins/hypocretins (OX) and melanin-concentrating hormone (MCH) neurons located in the lateral hypothalamus seem to modulate different stages of the sleep–wake cycle. OX are necessary for wakefulness and MCH appears to regulate rapid eye movement sleep (REMS). Likewise, endocannabinoids, the endogenous ligands for cannabinoid receptors 1 and 2 (CB1R, CB2R), also modulate REMS in rats. Moreover, it has been shown that the activation of the CB1R in the lateral hypothalamus of rats excites MCH neurons while inhibiting OX neurons in *in vitro* preparations. Hence, we assessed the effects of 2-arachidonoylglicerol (2-AG, an endocannabinoid) in the lateral hypothalamus on the sleep–wake cycle of rats. We also utilized the CB1R inverse agonist AM251 to further support the involvement of this receptor, and we performed double immunofluorescence experiments to detect c-Fos, as a marker of neural activation, in OX and in MCH neurons to determine which neurons were activated. Our results indicate that 2-AG increases REMS through CB1R activation, and increases c-Fos expression in MCH neurons. These results suggest that endocannabinoid activation of the CB1R in the lateral hypothalamus, which activates MCH neurons, is one mechanism by which REMS is triggered.

1. Introduction

Orexins/hypocretins (OX) and melanin-concentrating hormone (MCH) are peptides synthesized by separate populations of neurons within the perifornical and lateral hypothalamic regions of the brain (Bittencourt et al., 1992; Broberger et al., 1998). OX and MCH peptides were initially described as appetite enhancers in rodents (Della-Zuana et al., 2002; Yamanaka et al., 1999); however, several subsequent studies have indicated that these peptides modulate the sleep–wake cycle (SWC). For example, it has been shown that OX neurons discharge maximally in wakefulness (W) while remaining silent in both slow wave sleep (SWS) and in rapid eye movement sleep (REMS) (Lee et al, 2005; Mileykovskiy et al., 2005). Likewise, pharmacological stimulation of the OX system increases W and reduces REMS (Akanmu and Honda, 2005; Hagan et al., 1999). It has also been shown that OX *knockout* (KO) mice exhibit a narcoleptic-like phenotype, which includes sudden transitions from W into REMS (Chemelli et al., 1999). In turn, MCH neurons have their maximal firing rate during REMS, moderate during SWS and absent during W (Hassani et al., 2009). Also, microinjection of the MCH peptide increases REMS and diminishes W (Verret et al., 2003). Moreover, MCH KO mice exhibit increased W and reduced sleep (Willie et al., 2008), and blocking MCH neurotransmission increases W while reducing SWS and REMS (Ahnaou et al., 2008). Likewise, using c-Fos immunoreactivity as a marker of neural activation, it has been observed that OX neurons are active in W (Modirrousta et al., 2005) while MCH neurons are active in REMS (Verret et al., 2003). In addition, we have observed that activation of the protease-activated receptor 1 (PAR1) in the lateral hypothalamus increases REMS, but, interestingly, such an effect is blocked by AM251 (Pérez-Morales et al., 2012), a cannabinoid receptor 1 (CB1R) inverse agonist. It is known that endocannabinoids (eCBs), the endogenous ligands for CB1R and cannabinoid receptor 2 (CB2R), have been implicated in sleep regulation and have been shown to be strong sleep-inducing

Abbreviations: OX, Orexins/hypocretins; MCH, melanin-concentrating hormone; CB1R, cannabinoid receptor 1; CB2R, cannabinoid receptor 2; SWC, sleep-wake cycle; W, wakefulness; SWS, slow wave sleep; REMS, rapid eye movement sleep; eCBs, endocannabinoids; 2-AG, 2-araquidonoylglicerol; AEA, anandamide; OLE, oleamide; PAR1, protease-activated receptor 1; FAAH, fatty acid amide hydrolase; PBS, phosphate-buffer-saline; PFH, paraformaldehyde; LDT, laterodorsal tegmental nuclei; PPT, pedunculopontine tegmental nuclei; PACAP, pituitary adenylyl cyclase-activating polypeptide; FTG, gigantocellular tegmental field; PnO, pontine reticular nucleus; ACEA, arachidonyl-2′-chloroethylamide; IL-1, interleukin-1.

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molecules. For example, cis-9, 10-octadecenoamide (oleamide, or OLE) was isolated from the cerebrospinal fluid of sleep-deprived cats (Lerner et al., 1994), and its systemic administration induces sleep in rats (Cravatt et al., 1995). Moreover, OLE restores sleep in rats that exhibit a reduced sleep amount as a result of early maternal separation (Reyes Prieto et al., 2012). Likewise, Santucci et al. (1996) reported that systemic administration of SR141716A, a CB1R inverse agonist, increases W and decreases both SWS and REMS in rats. Acute (Murillo-Rodríguez et al., 2001), subchronic (Herrera-Solís et al., 2010) or intrahippocampal (Rueda-Orozco et al., 2010) administration of the eCB, anandamide (AEA), increases REMS in rats, through CB1R activation. Mice lacking fatty acid amide hydrolase (FAAH), the enzyme responsible for degrading AEA and OLE (Cravatt et al., 1996), have increased sleep and reduced W (Huitrón-Reséndiz et al., 2004). Despite all this information, an interaction between the peptidergic and endocannabinergic systems to induce sleep has not been described. There is evidence indicating that WIN55 212 2, a potent CB1R synthetic agonist, depolarizes MCH-ergic neurons and hyperpolarizes OX neurons in *in vitro* preparations (Huang et al., 2007), suggesting that eCBs could interact with these hypothalamic neurons to regulate sleep. Based on this literature, we decided to study the effect of 2-arachidonoylglicerol (2-AG, a potent eCB) as a hypnogenic molecule, when administered into the lateral hypothalamus of rats. We also utilized the CB1R inverse agonist AM251, to determine if 2-AG's effects were mediated by CB1R activation. Finally, to study if 2-AG's effects resulted from the interaction with OX and MCH neurons, we explored the expression of c-Fos, as a marker of neural activation, in such neurons after 2-AG administration.

2. Materials and method

2.1. Subjects

Seventy-two male Wistar rats (250–300 g) provided by the vivarium of the School of Medicine, Universidad Nacional Autónoma de México (UNAM), were housed individually in Plexiglas cages (CIPQUIM, México) with wood chip bedding, and maintained under a reversed dark-light cycle (12:12; lights ON at 8: 00 PM) with constant environmental temperature (23 ± 1 °C) and food and water *ad libitum*. Animals remained in their home cages throughout the experiments, including the SWC recording, and were handled according to the Mexican Official Standard on "Technical specifications for the production, use and care of laboratory animals" (NOM-062-ZOO-1999). Additionally, this study was approved by the Public Health Service (PHS) Policy of Humane Care and Use of Laboratory Animals, and the Ethics Committee guidelines of the School of Medicine (UNAM). All possible efforts were made to minimize the number of animals used, as well as their potential suffering.

2.2. Drugs and chemicals

2-arachidonoylglicerol (2-AG) (Catalogue Number: 62160) and CB1R inverse agonist AM251 [1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidynil-1H-pyrazole-3-carboxamide] (Catalogue Number: 71670) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Vehicle for 2-AG and AM251 was a solution of Dimethyl sulfoxide (100%, DMSO), obtained from Sigma Aldrich (Catalogue Number: 673439; St. Louis, MO, USA). Paraformaldehyde (Catalogue Number P6148) and bovine albumin were purchased from Sigma-Aldrich (Catalogue Number A-7030; St. Louis, MO, USA). 4′, 6-Diamidino-2 -Phenylindone, Dihydrochloride (DAPI) was obtained from Invitrogen (Catalogue Number D3571; San Diego, CA, USA). DAKO fluorescence mounting medium was purchased from Dako North America, Inc. (Code S3023).

2.3. Antibodies

Orexin-A (C-19) goat polyclonal IgG (Catalogue Number sc-8070), proMCH (E-16) goat polyclonal IgG (Catalogue Number sc-14507) and c-Fos (H-125) rabbit polyclonal IgG (Catalogue Number sc-7202), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor® 488 Donkey Anti-rabbit IgG (H + L) (Catalogue Number A-21206) and Alexa Fluor® 555 Donkey Anti-Goat IgG (H + L) (Catalogue Number A-21432) were obtained from Invitrogen (San Diego, CA, USA).

2.4. Sleep–wake cycle experiments

2.4.1. Electroencephalographic and electromyographic recordings

Rats under anesthesia (a mixture of ketamine, 66 mg/kg; xylazine, 0.26 mg/kg; and acepromazine, 1.3 mg/kg), were stereotaxically implanted with a set of electrodes for polysomnographic recordings, that consisted of two stainless-steel screw electrodes (0–80 × 1/16, Plastics One Inc., VA, USA) placed in the frontal bones in order to ground the animal; a bipolar stainless-steel Teflon-covered wire electrode (0.013″, A-M Systems Inc., WA, USA) was placed in the hippocampus (P = 4.0; L = ±2; V = 2.5, reference from Bregma), according to Paxinos and Watson atlas (2007), for electroencephalographic recording (EEG), since theta rhythm is easily recorded from this structure (Prospéro-García et al., 1993) and allows us to differentiate between the SWC stages, as we have done previously (Pérez-Morales et al., 2012); and two additional twisted stainless-steel Teflon-covered wire (0.013″, A-M Systems, WA, USA) electrodes were placed into the rats' neck, for electromyographic recording (EMG). This standard set of electrodes to record the SWC was affixed to the rats' skull with dental acrylic. 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. Sleep recordings were captured and analyzed using the commercially available Icelus® software (Mark Opp, Ann Arbor, MI, USA). Three SWC phases were visually identified and scored offline: W (EEG: low voltage and high frequency activity, or mixed activity; EMG: high activity), SWS (EEG: high voltage and low frequency activity; EMG: low activity), and REMS (EEG: predominantly low voltage and high frequency activity; EMG: absent activity).

2.4.2. Guide cannulae implantation

During the same surgical procedure mentioned in Section 2.4.1, rats were implanted with a couple of stainless steel guide cannulae (23-gauge × 1.1 cm, Plastics One) aimed bilaterally at the lateral hypothalamus (P = 1.8; L = ±2; V = 6.6; reference to Bregma) (Paxinos and Watson, 2007), to administer drugs. Guide cannulae were implanted and affixed to the rats' skull with dental cement and sealed with stylets to maintain their patency. The stylets, as well as the 30-gauge injectors (Plastics One) used to infuse the drugs, protruded 1 mm from the end of the guide cannulae. All animals were allowed to recover from surgery for 10 days, during which they remained under a reversed dark-light cycle in the conditions mentioned in Section 2.1 until the end of the experiment, and then gently habituated to manipulation for three days, 1 h each, to reduce stress, before the beginning of drug administrations.

2.4.3. Drug administration

Intra-hypothalamic administration of drugs was conducted at the onset of the dark phase of the cycle, for both SWC and the immunofluorescence experiments. In all cases, drugs were dissolved in a final volume of 0.5 μl, even when combining 2-AG with AM251, and were administered into the lateral hypothalamus of rats with the aid of an infusion pump (kd Scientific®, Holliston, MA, USA), at a rate of 0.1 μl per min, through the 30-gauge injectors mentioned in Section 2.4.2. Injections were performed while animals were