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Microinjection of melanin concentrating hormone into the lateral preoptic area promotes non-REM sleep in the rat

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ABSTRACT

The ventrolateral preoptic area (VLPO) has been recognized as one of the key structures responsible for the generation of non-REM (NREM) sleep. The melanin-concentrating hormone (MCH)-containing neurons, which are located in the lateral hypothalamus and incerto-hypothalamic area, project widely throughout the central nervous system and include projections to the VLPO. The MCH has been associated with the central regulation of feeding and energy homeostasis. In addition, recent findings strongly suggest that the MCHergic system promotes sleep. The aim of the present study was to determine if MCH generates sleep by regulating VLPO neuronal activity. To this purpose, we characterized the effect of unilateral and bilateral microinjections of MCH into the VLPO on sleep and wakefulness in the rat. Unilateral administration of MCH into the VLPO and adjacent dorsal preoptic area did not modify sleep. On the contrary, bilateral microinjections of MCH (100 ng) into these areas significantly increased light sleep (LS, 39.2 ± 4.8 vs. 21.6 ± 2.5 min, $P < 0.05$) and total NREM sleep (142.4 ± 23.2 vs. 86.5 ± 10.5 min, $P < 0.05$) compared to control (saline) microinjections. No effect was observed on REM sleep. We conclude that MCH administration into the VLPO and adjacent dorsal lateral preoptic area promotes the generation of NREM sleep.

1. Introduction

Since von Economo's classical report, a number of studies have confirmed the proposal that the preoptic area of the hypothalamus (POA) is involved in the generation of non-REM (NREM) sleep [27,34]. The use of several experimental approaches, including restricted lesions, electrical and chemical stimulation procedures, and the study of neuronal activity by unit recordings or *c-fos* expression have allowed the identification of POA subregions, such as the median preoptic nucleus (MnPN) and the ventrolateral preoptic region (VLPO), as the critical structures responsible for NREM sleep occurrence [2,6–8,18,29]. In the rat, the VLPO has been identified as a cluster of neurons that show Fos immunoreactivity during sleep. Among other brain areas, these neurons project to the waking-related histaminergic tuberomamillary nucleus (TMN) [25,26].

Melanin concentrating hormone (MCH), a 19 aminoacid cyclic peptide initially described in teleost fish [11], is a neuromodulator synthesized in neurons localized in the lateral hypothalamus and incerto-hypothalamic area of mammals [3,32]. The MCHergic system projects widely throughout the central nervous system (CNS) and sends relatively dense projections to the VLPO [3]. MCH acts through G-proteins via two types of receptors, MCHR1 and MCHR2. The former is the only functional subtype in rodents [28].

Classically, MCH has been involved in the central regulation of feeding and energy homeostasis [23]. However, we and other groups have demonstrated the importance of this neuropeptide in the regulation of sleep (for review, see [30]). Recently, we characterized the role of MCH in the modulation of sleep and wakefulness (W) in different brain areas, such as the dorsal raphe nucleus (DRN) and the lateral basal forebrain of the rat, as well as the nucleus pontis oralis (NPO) of the cat. Microinjection of MCH into these nuclei resulted in an increase in REM sleep [14,31]. To date, no attempts have been made to determine whether MCH intervenes in the VLPO-induced generation of NREM sleep. Thus, the aim of the present study was to define the effects of MCH microinjection into the VLPO on sleep variables in the rat.

2. Materials and methods

Fourteen adult male Wistar rats (240–260 g) were employed in this study. All rats were used in strict accordance with the "Guide

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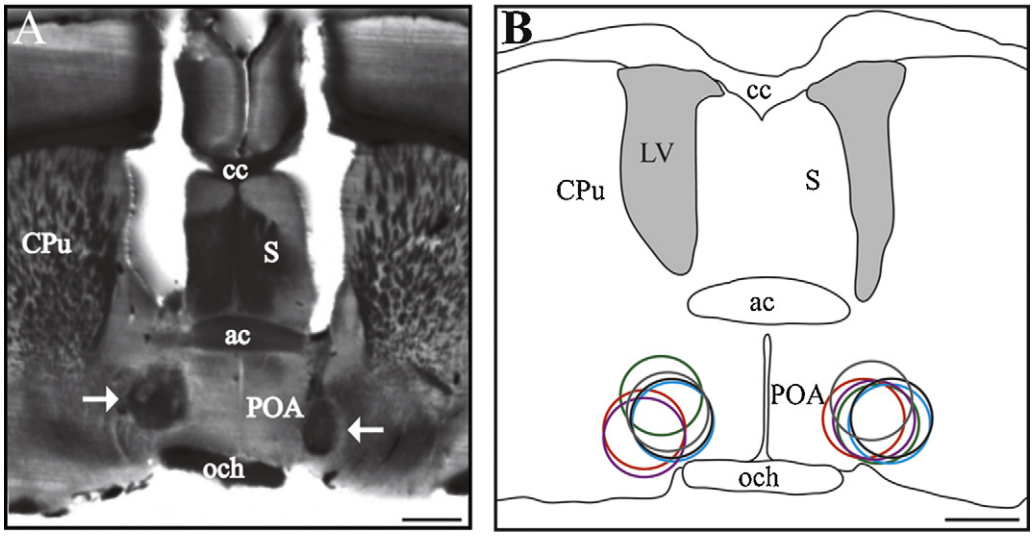

Fig. 1. Microinjections sites. (A) Representative photomicrograph of a coronal section at the level of the VLPO. The microinjection sites are recognizable by the cannulae track lesions. (B) Schematic drawing of a coronal section showing the approximate microinjections sites of animals with bilateral administration of MCH (one color per animal). ac, anterior commisure; cc, corpus callosum; CPu, caudate putamen; och, optic chiasm; S, septum. Calibration bars: 1 mm.

to the care and use of laboratory animals" (8th edition, National Academy Press, Washington, DC, 2011) and the Institutional Animal Care Committee approved the experimental procedures. In addition, adequate measures were taken to minimize the pain, discomfort and stress of the animals, and all efforts were made to use the minimal number of animals necessary to produce reliable scientific data.

Rats were implanted under ketamine/xylazine anesthesia (90/5 mg/kg, i.p.) with cortical electroencephalogram (EEG) electrodes and dorsal neck muscle electromyogram (EMG) electrodes for the assessment of sleep and W states; these electrodes were soldered to a nine-pin connector. In addition, unilateral ($n = 7$) or bilateral ($n = 7$) guide cannulae (gauge 26) were placed 2 mm above the VLPO. The coordinates for the VLPO were AP −0.6 mm, *L* 0.9 and *H* −8.9 mm from Bregma [20]. The connector and cannulae were cemented to the skull using dental acrylic. Immediately after surgery, a dummy cannulae was inserted into the guide cannulae to prevent their blockage.

MCH (human, mouse, rat; Phoenix Pharmaceuticals Inc., Belmont, CA, #070-47) was diluted in sterile saline to obtain a final concentration of 0.25 and 0.5 μg/μl. Aliquots for the doses employed were prepared and frozen at −20 °C, and thawed immediately before use.

The animals were housed individually in a temperature-controlled room (23 ± 1 °C) in a soundproof chamber under a 12 h light/dark cycle (lights-on at 6.00 AM), with food and water ad libitum. Seven days after surgery, the animals were habituated to a sound-proof chamber fitted with slip rings and cable connectors and to the microinjection procedure. Standard recordings of the EEG and EMG signals were performed.

After three consecutive stable recording sessions of 5 h (<10% fluctuation in sleep and W parameters among recordings), experimental microinjections were initiated. At the beginning of the recording sessions, MCH (50 or 100 ng in 0.2 μl of saline/side) or the same volume of saline was microinjected into the VLPO during a period of 2 min, with an injection cannulae (28 gauge), which extended 2 mm beyond the guide cannulae.

To maximize the effect of MCH on sleep, the experiments were performed during the dark (active) phase; microinjections were carried out immediately before dark onset, at approximately 6 PM. The recording sessions began immediately after the microinjection procedures and lasted for 5 h. In bilateral implanted rats, each animal received three microinjections: saline, 50 ng of MCH, and 100 ng of MCH in a counterbalanced design, while unilaterally implanted rats received only saline and 100 ng of MCH. These same doses were used in previous studies of our laboratory [13,14,16]. Only one microinjection was performed prior to each recording session, and no further microinjection experiments were conducted during the following two days.

At the end of the experimental sessions, animals were euthanized with sodium pentobarbital (60 mg/kg), perfused with 4% paraformaldehyde, and their brains were removed for histological processing. Thereafter, the brains were cut in 150 μm coronal sections with a vibratome.

Polysomnographic data were acquired and stored in a microcomputer for further analysis using Spike 2 software (CED, Cambridge, UK). The states of sleep and wakefulness (W) were

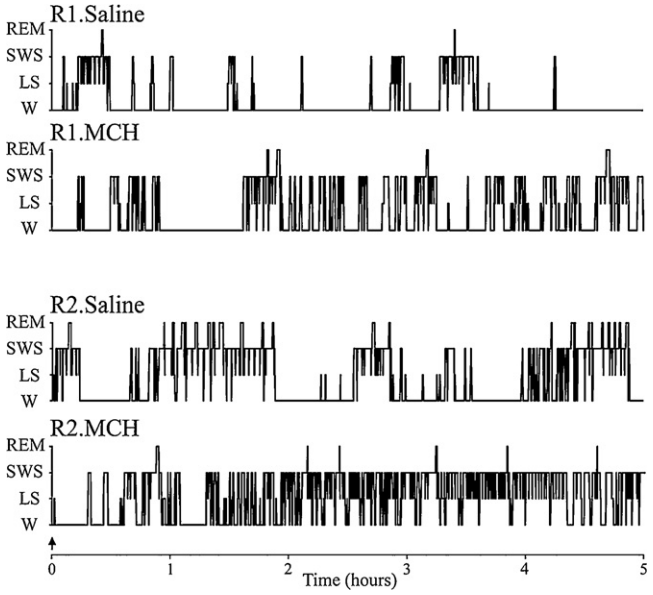

Fig. 2. Representative hypnograms after saline and MCH (100 ng) microinjections. Two example animals (R1 and R2) are shown. The arrow indicates the time of the microinjections. MCH (100 ng) microinjection resulted in an increase in NREM sleep time.

determined in 10 s epochs as either W (low voltage fast waves in frontal cortex, a mixed theta rhythm (4–7 Hz) in occipital cortex and relatively high electromyographic activity); light sleep (LS, high voltage slow cortical waves interrupted by low voltage fast electroencephalographic activity); slow wave sleep (SWS, continuous high amplitude slow frontal and occipital waves combined with a reduced electromyogram) and REM sleep (low voltage fast frontal waves, a regular theta rhythm in the occipital cortex, and a silent electromyogram except for occasional myoclonic twitching) [19].

Total time spent in W, LS, SWS, NREM sleep (LS + SWS) and REM sleep, as well as the duration and the number of episodes over a 5 h-recording period, were analyzed. Sleep latencies were also included in the analysis. Besides, the time spent in each state was analyzed separately the first hour (usually influenced by the microinjection procedure) and the remaining recording time into 2 blocks of 2 h: 2–3 and 4–5.

All values are presented as mean ± S.E.M. The experimental design was a within-subject design where statistical significance of the differences among groups (MCH 0, 50 and 100 ng) was evaluated utilizing a one-way repeated measures ANOVA. Post hoc comparisons was performed with the Fisher's test when ANOVA indicated significance ($P < 0.05$). To discard any disturbance of the animals due to microinjection procedures, comparisons between baseline, unilateral saline and bilateral saline microinjection experiments were also performed by means of one-way ANOVA.

3. Results

In eleven animals the tip of the microinjection cannulae was placed in the region of the VLPO; in most of the animals also included an adjacent dorsal region of the lateral preoptic area. Fig. 1A shows a photomicrograph illustrating the bilateral sites of microinjection at the level of the VLPO in one representative animal. Fig. 1B summarizes the sites of the successful bilateral microinjections. Three animals were excluded because the cannulae were misplaced.

The time spent in wakefulness and sleep in baseline experiments were: W, 188.1 ± 8.6 min; LS, 26.1 ± 3.3 min; SWS, 72.0 ± 7.4 min; NREM, 98.1 ± 9.4 min; REM, 13.8 ± 1.7 min. There were no statistical difference in sleep and wakefulness parameters when baseline, unilateral or bilateral saline microinjections targeted to the VLPO were compared (see saline microinjections values in Tables 1 and 2).

After unilateral microinjections of MCH (100 ng) no significant differences were found in any of the parameters studied compared with saline microinjections (Table 1).

Table 2 shows sleep and W parameters after bilateral local delivery of MCH or saline into the VLPO (and adjacent dorsal regions). Although no significant effects were observed after microinjections of 50 ng of MCH, 100 ng of MCH increased the time spent in total NREM sleep (LS + SWS), as shown in the representative hypnograms in Fig. 2 and in Table 2. Additionally, the time spent in LS, as well as the number of LS and SWS episodes, were augmented after local administration of 100 ng MCH ($P < 0.05$, Table 2). Compared with saline microinjections values, no differences were found in any of the REM sleep variables (Table 2). In contrast, after treatment with 100 ng MCH, there was a clear tendency for a reduction of the time spent in W ($P = 0.06$), which was related to a significant decrease in the duration and number of W episodes ($P < 0.05$). Interestingly, no significant effect on sleep and W were observed during the first hour, that it is usually affected by the microinjection procedure. On the contrary, while an increase in LS was observed during 2–3 and 4–5 h blocks, a significant decrease in W was evident during the 4–5 h block of the recordings (Fig. 3). NREM sleep latency did not vary significantly after local delivery of MCH into the VLPO (Table 2).

Table 1
Effects of unilateral microinjections of MCH into the VLPO on sleep and wakefulness.

	Saline	MCH (100 ng)
Wakefulness		
Time (min)	189.0 ± 6.3	208.6 ± 13.8
Number of episodes	59.4 ± 8.5	47.5 ± 3.4
Episodes duration (min)	3.6 ± 0.5	4.6 ± 0.5
Light sleep		
Time (min)	24.9 ± 3.5	19.3 ± 2.2
Number of episodes	99.2 ± 9.7	81.8 ± 8.5
Episodes duration (min)	0.2 ± 0.0	0.2 ± 0.0
Slow wave sleep		
Time (min)	72.7 ± 4.0	58.2 ± 8.9
Number of episodes	80.4 ± 7.6	64.5 ± 8.0
Episodes duration (min)	0.9 ± 0.0	0.9 ± 0.0
NREM sleep (LS + SWS)		
Time (min)	97.5 ± 0.25	77.4 ± 10.3
Latency	6.4 ± 0.6	7.4 ± 3.6
REM sleep		
Time (min)	13.5 ± 2.2	14.0 ± 3.9
Number of episodes	12.8 ± 2.4	14.3 ± 2.9
Episodes duration (min)	1.1 ± 0.2	1.0 ± 0.2
Latency	16.1 ± 4.8	15.2 ± 4.0

The data were obtained from experiments conducted in five animals.

It should be noted that microinjections of MCH did not produce abnormal behaviors or movements during W. Moreover, the EEG and EMG did not show atypical activity either during sleep or W.

4. Discussion

In the present report, we examined the role of MCH on the regulation of sleep, after it was microinjected into the VLPO. In these experiments, MCH (50 and 100 ng) were microinjected into the VLPO of animals prepared for chronic sleep recordings; the microinjections also included adjacent dorsal regions of the lateral preoptic area. We found that bilateral microinjections of the neuropeptide produced an increase in the time spent in NREM sleep, whereas REM sleep time as well as the number and duration of REM sleep episodes were not affected.

In the present study, we performed intracerebral microinjection of MCH in a volume of 0.2 μl, similarly to previous studies of our laboratory [14,15]. Notably, the same volume of methylene blue has been shown to diffuse approximately 500 μm in the CNS [17]. Therefore, although adjacent dorsal regions were affected by the microinjections, we believe that the microinjection of MCH did not extend far beyond the VLPO. In fact, MCH administration into the neighbor nucleus of the horizontal limb of the diagonal band of Broca increased the time that the animals spent in REM sleep, but did not affect NREM sleep [12]. Despite this result, we cannot rule out the possibility that the drug could have potentially affected the medial preoptic area, which are also linked to sleep generation [10].

Bilateral microinjections of MCH into the VLPO selectively enhanced NREM sleep, mainly by increasing the number of LS and SWS episodes; no effects were observed on REM sleep. The increase of NREM sleep was observed only with the largest dose (100 ng). On the contrary, unilateral microinjection of MCH at the same dose failed to promote sleep. We believe that unilateral microinjection of this regulatory neuropeptide did not produce a significant change in sleep due to the compensatory activity of the contralateral VLPO. These results suggest that these bilateral structures should be regulated in tandem in order to generate NREM sleep.

Previous evidence suggests that MCH promotes primarily REM sleep in laboratory animals [30]. However, the MCHergic system

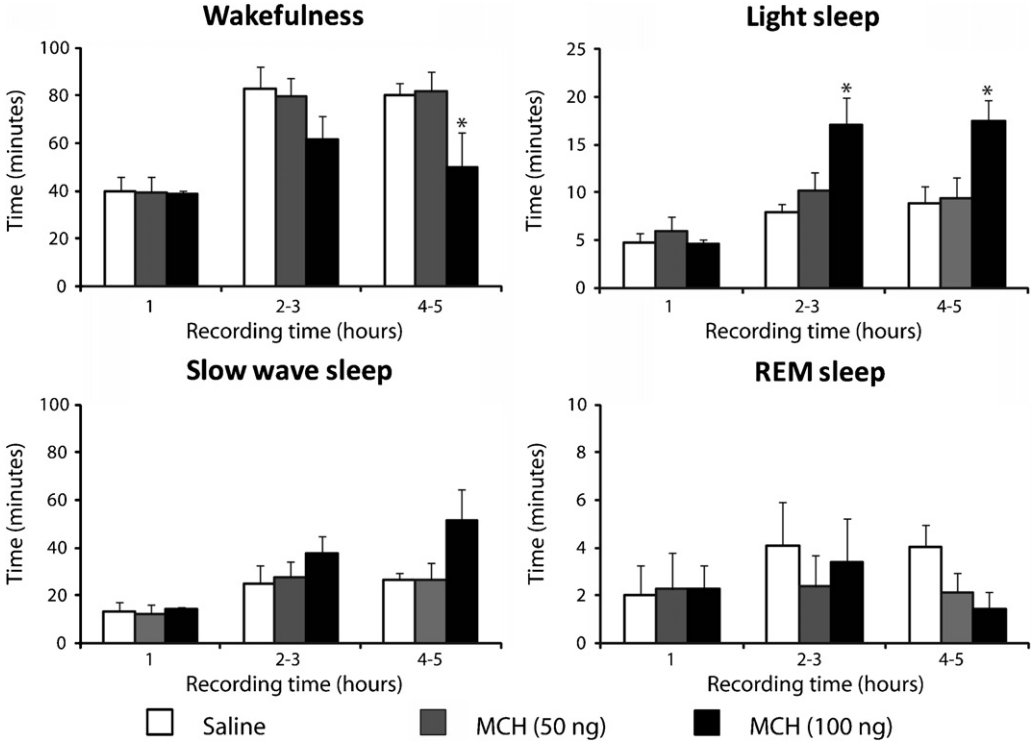

Fig. 3. Effects on sleep and wakefulness of MCH microinjections into the VLPO. Graphic chart of the mean time spent in wakefulness, light sleep, slow wave sleep (SWS) and REM sleep after local administration of saline and MCH (50 and 100 ng) during the first hour and the following 4 h analyzed in blocks of 2 h: 2–3 and 4–5. Group mean differences determined by one way ANOVA repeated measures and Fisher as post hoc test; significant differences between MCH 100 ng and saline microinjections are indicated by asterisks ($^{*}P < 0.05$).

seems to be also involved in the promotion of NREM sleep. In this respect, Hassani et al. demonstrated that the firing rate of the MCHergic neurons reach its zenith during REM sleep [9]. However, MCHergic neurons also increase their firing rate during NREM sleep when compared with W. In addition, behavioral studies in MCH knockout mice have shown that during basal conditions, the amount of NREM sleep was reduced compared with wild-type littermates, while REM sleep time was unchanged. In response to fasting, MCH knockout mice exhibited marked hyperactivity, accelerated weight loss, decrease in NREM sleep and an exaggerated decrease in REM sleep. Thus, MCH knockout mice adapt poorly to fasting; these data support a role for MCH in vigilance state

Table 2
Effects of bilateral microinjections of MCH into the VLPO on sleep and wakefulness.

	Saline	MCH (50 ng)	MCH (100 ng)	*F*	*P*		
				(2,10)	ANOVA	Post hoc	
						*	#
Wakefulness							
Time (min)	203.4 ± 13.2	201.3 ± 16.5	150.4 ± 23.9	3.7	0.062		
Number of episodes	42.3 ± 5.0	53.5 ± 8.9	73.5 ± 9.9*	4.3	0.044	0.016	0.091
Episodes duration (min)	5.3 ± 0.8	4.5 ± 1.9	2.2 ± 0.4*#	7.5	0.010	0.004	0.021
Light sleep (LS)							
Time (min)	21.6 ± 2.5	25.6 ± 5.0	39.2 ± 4.8*#	7.5	0.010	0.004	0.017
Number of episodes	78.0 ± 7.3	91.0 ± 15.8	136.7 ± 19.0*#	7.7	0.009	0.004	0.016
Episodes duration (min)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.16	0.858		
Slow wave sleep (SWS)							
Time (min)	65.0 ± 10.3	66.4 ± 12.9	103.2 ± 19.7	2.5	0.128		
Number of episodes	57.7 ± 6.6	65.5 ± 11.9	103.7 ± 17.9*#	6.1	0.018	0.009	0.022
Episodes duration (min)	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.2	0.1	0.908		
NREM sleep (LS + SWS)							
Time (min)	86.5 ± 10.5	92.0 ± 17.1	142.4 ± 23.2*#	4.4	0.043	0.023	0.036
Latency	18.9 ± 6.4	15.8 ± 4.9	6.0 ± 4.9	0.5	0.626		
REM sleep							
Time (min)	10.1 ± 2.9	6.8 ± 2.3	7.1 ± 3.1	0.5	0.631		
Number of episodes	12.5 ± 3.0	6.8 ± 4.7	4.4 ± 1.0	1.6	0.247		
Episodes duration (min)	0.7 ± 0.1	0.7 ± 0.2	0.9 ± 0.2	0.2	0.857		
Latency	46.2 ± 21.4	131.4 ± 48.5	62.1 ± 11.2	1.0	0.403		

Significant differences between MCH (100 ng) vs. saline, and MCH (100 ng) vs. MCH (50 ng) are indicated by asterisk (*) or numeral (#), respectively. One way repeated measures ANOVA and Fisher's post hoc test. The data were obtained from experiments conducted in six animals.

regulation in response to changes in energy homeostasis [35]. It is known that MCH decreases temperature, heart rate, and metabolic rate and enhances the parasympathetic/sympathetic tone ratio [23]. Given the profile of the activity of the MCHergic neurons, it is possible that these actions are maximally expressed during NREM sleep.

The effect of MCH on NREM sleep is site-specific. In fact, microinjections of MCH into the DRN during the light phase produce a small increase in NREM sleep (approximately 9% over the control values) and a larger increase in REM sleep (approximately 70% over the control values). On the contrary, microinjections of MCH into the NPO of the cat produced a specific increase in REM sleep time [31]. When MCH reach widespread regions as in intracerebroventricular administration during the dark phase, both NREM and REM sleep are increased (up to 70% and 300% of the control values, respectively) [33]. Additionally, subcutaneous administration of MCHR1 antagonists decreased the time the animals spent both in NREM and REM sleep [1].

The present data are consistent with several reports showing that the VLPO specifically generates NREM sleep [7,21,24]. Thus, substances administrated into this nucleus can modify NREM sleep but not REM sleep.

The VLPO is composed predominantly of GABAergic and galaninergic neurons, which project to different areas associated with the generation of W [25]. In turn, the VLPO receives many projections from the reticular activating system, which either directly or indirectly decreases VLPO neuronal activity and facilitate W [4,5]. Although the presence of MCHR1 has been demonstrated in the lateral preoptic area [22], the neuronal phenotype and specific cellular localization of MCHR1 in the VLPO remains unknown. Our working hypothesis is that the MCHR1 may localize presynaptically in waking-related axons (serotonergic, noradrenergic and/or hypocretinergic) that innervate the VLPO and inhibit sleep. Therefore, MCH would promote NREM sleep by inhibiting the release of the wake-promoting neurotransmitters into the VLPO.

5. Conclusions

The present study demonstrates that MCH can induce NREM sleep by modulation of the VLPO neuronal activity.

Acknowledgments

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