Serotonergic System in the Central Nucleus of Amygdala Mediates Cannabidiol-Induced Sleep Alteration

Pei-Lu Yi[1], Yi-Tes Hsiao[2], Chon-Haw Tsai[3], Tong-Rong Jan[2], Chin-Yu Lu[2], Fang-Chia Chang*[,2,3,4]

[1]*Department of Medical Technology, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli, Taiwan*

[2]*Department of Veterinary Medicine, National Taiwan University, Taipei, Taiwan*

[3]*Neuroscience Laboratory, Department of Neurology, China Medical University Hospital, Taichung, Taiwan*

[4]*Graduate Institute of Acupuncture Science, College of Chinese Medicine, China Medical University, Taichung, Taiwan*

Abstract: Cannabidiol (CBD) is one of the psycho-inactive constituents of marijuana, the *Cannabis sativa*. The pharmacological property of CBD, especially the anxiolytic effect, is significant in the therapeutic purposes. The central nucleus of amygdala (CeA) plays a key role in the anxiety and its related behavioral responses (e.g. sleep-wake activity), and serotonin is one of the major mediators. However, the sleep-wake effect of CBD remains unclear. This study was designed to elucidate the effects of CBD on sleep-wake alteration and the involvement of serotonin in the CeA. Administrations of 5-hydroxytryptamine (5-HT), 5-HT$_{1A}$ receptor partial agonist (buspirone), 5-HT$_2$ antagonist (ritanserin), cannabinoid CB$_1$ receptor agonist (ACEA), or CB$_1$ antagonist (AM-251) were employed to elucidate the action of CBD on CeA presynaptic CB$_1$ receptors, serotonergic activity and the subsequent sleep alteration. We found that microinjection of CBD into the CeA prior to the beginning of the light period dose-dependently decreased slow wave sleep (SWS) with limited effect on rapid eye movement sleep (REMS). CBD-induced SWS suppression during the light period could be mimicked by administering serotonin into the CeA. Buspirone and ritanserin dose-dependently blocked CBD-induced SWS decrease. Furthermore, administration of AM-251 exhibits similar effect as that of CBD on sleep-wake activity, and the CBD-induced SWS decrease was partially blocked by ACEA. These observations suggest that CBD acting on the CeA neurons decreases SWS during the light phase, which is at least partially mediated by the consequence of antagonizing presynaptic CB$_1$ receptors, enhancing serotonin release from the presynaptic terminals and subsequently acting on the postsynaptic 5-HT$_2$ receptors.

Keywords: Cannabidiol, serotonin, amygdala, sleep.

INTRODUCTION

The abuse of marijuana (the *Cannabis sativa* preparation), which is one of the most used illicit drugs worldwide, has drawn much attention. More than 60 active compounds (cannabinoids) have been identified in cannabis [1]. Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) are two major constituents of the *Cannabis sativa* [2,3]. Two cannabinoid receptors, CB$_1$ and CB$_2$, have been identified to date. Both CB$_1$ and CB$_2$ receptors are coupled to the pertussis toxin-sensitive G-protein, G$_{i,o}$, which inhibits adenylate cyclase and subsequently reduces the conversion of ATP to cyclic AMP (cAMP) [4]. Activation of CB$_1$ receptor produces psychoactive effects which are similar to the phenomenon after ingestion of cannabis, whereas activation of the CB$_2$ receptor does not cause the psychoactive effect [5]. The CB$_1$ receptor is highly expressed in the basal ganglia, cerebellum, hippocampus and olfactory cortex, and its expression is moderate in the cerebral cortex, amygdala, septum and brain stem [6-8]. In contrast, CB$_2$ receptor mostly expresses in the peripheral immune cells [9], modulates the

release of cytokines, and is responsible for the inflammation and the regulation of the immune system [10]. Δ^9-THC has a strong binding affinity for the central CB$_1$ receptors and produces psychoactive properties, including anxiogenic effect [5]; whereas CBD exhibits a low binding affinity to the CB$_1$ receptors and has a non-psychoactive property [5,11]. Therefore, the central nervous system (CNS) pharmacological properties of CBD are more predominant in the therapeutic purposes due to its non-psychoactivity [11]. The mechanisms of cannabinoids are well investigated for Δ^9-THC [1,4,5], however the mode of therapeutic effect of CBD is less established.

The effects of CBD, including the anxiolytic action and the influence of spontaneous sleep-wake activity, have attracted our interest, since anxiety could cause sleep disturbance and the sleep perturbation further deteriorates the syndrome of anxiety. The central nucleus of amygdala (CeA) plays a key role in the sleep-wake regulation [12,13], in addition to the emotional processing [14] and anxiety-related physiological and behavioral responses, such as the fear-potentiated startle [15]. Serotonin is one of the major neurotransmitters in the CeA, and the highest density of serotonergic fibers is observed in the central nucleus, paralaminar nucleus and anterior parts of the amygdaloid [16]. This observation suggests that the serotonergic system has substan-

*Address correspondence to this author at the Department of Veterinary Medicine, National Taiwan University, No. 1, Sec. 4., Roosevelt Road, Taipei 106, Taiwan; Tel: +886-2-3366-3883; Fax: +886-2-2366-1475; E-mail: fchang@ntu.edu.tw

tial influence on the ongoing activities of the amygdaloid complex. The CB_1 receptor generally locates on the axon terminals and leads to inhibit the neurotransmitter release [17]. It has been reported that CB_1 receptors and 5-HT transporters are co-distributed in the amygdala, implicating that the CB_1 receptor exists on the presynaptic terminals of serotonergic fibers and the activation of CB_1 receptor causes a reduction of serotonin release in amygdala [18]. Δ^9-THC has been demonstrated to increase stage 4 sleep [19] and decrease rapid eye movement (REM) sleep [20]. As for the CBD, intraperitoneal injection of a low dose (20 mg/kg) of CBD decreases slow-wave sleep latency in the rats, and a high dose (40 mg/kg) of CBD significantly increases slow wave sleep (SWS) [21]. REM sleep is not modified by CBD [21]. However, it has been reported that intracerebroventricular (ICV) administration of CBD during the light (rest) period increases wakefulness and decreases REM sleep in the rats [22], suggesting that the sleep-wake regulation of CBD and its underlying mechanisms remain ambiguous and limited. Recently, an interesting finding indicated that CBD displays a high potency of antagonizing CB_1 receptors [23,24]. Therefore, we designed the present study to elucidate the sleep-wake regulation of CBD when directly administered CBD into the CeA, to determine whether the action of CBD is mediated by antagonizing the presynaptic CB_1 receptors on the serotonergic fibers, and to demonstrate the involvement of serotonergic system in the CeA.

MATERIALS AND METHODOLOGY

Substances

Stock solutions of serotonin and buspirone (Tocris, Bristol, UK) were dissolved in pyrogen-free saline (PFS). AM-251 (Tocris) was dissolved in PFS with 0.5 % dimethyl sulfoxide (DMSO). CBD and ritanserin were dissolved in 1 % ethanol, and ACEA (Tocris) were dissolved in 10 % ethanol. These stock solutions were stored at -20 °C until used. The doses of the substances used in these experiments were as follows: for CBD, 0.5 and 1.0 µg; for buspirone, 0.5, 2.0 and 4.0 µg; for ritanserin, 5.0 and 10.0 µg; for serotonin, 3.0 and 10.0 µg; for ACEA, 100.0 and 300.0 pmol (36.6 and 109.8

ng, respectively); and 100.0 ng AM-251. The volume of total injection was 2 µl.

Animals

Male Wistar rats (250 - 300 g; National Laboratory Animal Breeding and Research Center, Taiwan) were used in these experiments. These animals were anesthetized (ketamine/xylazine; 87/13 mg/kg), and injected with analgesic (morphine) and antibiotic (penicillin G benzathine). Rats were surgically implanted with three EEG screw electrodes (on the right hemisphere of the frontal and parietal lobes and the left hemisphere of the occipital lobe) and a microinjection guide cannulae directed into the CeA of the left hemisphere (AP, -2.8 mm from bregma; ML, 4.2 mm; DV, 7.8 mm). The coordinates were adopted from the Paxinos and Waton rat altas [25]. Insulated leads from EEG electrodes were routed to a Teflon pedestal (Plastics One, Roanoke, VA). The Teflon pedestal was then cemented to the skull with dental acrylic (Cranioplastic cement and Cyanoacrylate gel, Plastics One, Roanoke, VA). The incision was treated topically with polysporin (polymixin B sulfate – bacitracin zinc) and the animals were allowed to recover for seven days prior to the initiation of experiments. The rats were housed separately in individual recording cages in the isolated room, in which the temperature was maintained at 23 ± 1 °C and the light:dark rhythm was controlled in a 12:12 h cycle (40 Watt x 4 tubes illumination). Food and water were available *ad libitum*. All procedures performed in this study were approved by the National Taiwan University Animal Care and Use Committee.

On the second postsurgical day, rats were connected to the recording apparatus (see later) *via* a flexible tether. The location of the microinjection cannuae was confirmed by injecting 0.5 % trypan blue dye at the end of experiment. The recording data could be included for the subsequent analyses only when the injection target has been confirmed inside the CeA in a rat (Fig. **1**). Animals were habituated by daily handling and injections of PFS timed to coincide with scheduled experimental administrations.
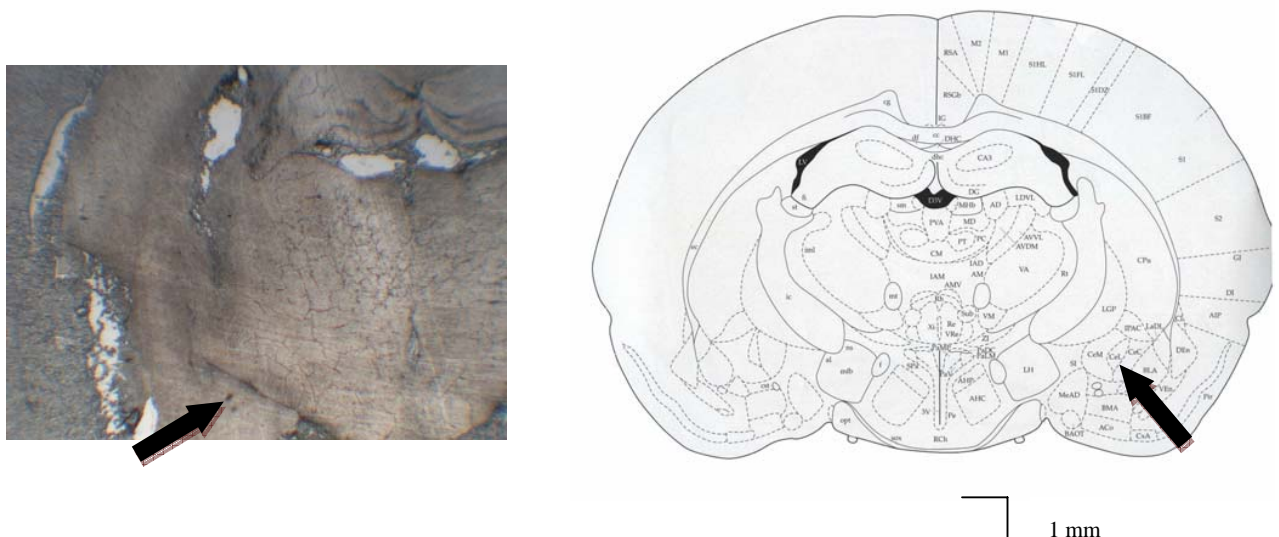

Fig. (1). The location of microinjection cannuae. Arrows point out the stain of trypan blue injected from the microinjection cannuae.

Apparatus and Recording

Signals from the EEG electrodes were fed into an amplifier (Colbourn Instruments, Lehigh Valley, PA; model V75-01). The EEG was amplified (factor of 5,000) and its analog bandpass filtered between 0.1 and 40 Hz (frequency response: ±3 dB; filter frequency roll off: 12 dB / octave). Gross body movements were detected by custom-made infrared-based motion detectors (Biobserve GmbH, Germany), and the movement activity was converted to a voltage output which was digitized and integrated into 1-s bins. These conditioned signals (EEGs and gross body movements) were subjected to analog-to-digital conversion with 16-bit precision at a sampling rate of 128 Hz (NI PCI-6033E; National Instruments, Austin, TX). The digitized EEG waveform and integrated values for body movement were stored as binary computer files until subsequent analysis.

Postacquisition determination of vigilance state was done by visual scoring of 12-s epochs using custom software (ICELUS, M. R. Opp) written in LabView for Windows (National Instruments). The animal's behavior was classified as either SWS, rapid eye movement sleep (REMS), or waking based on previously defined criteria [26]. Briefly, SWS is characterized by large-amplitude EEG slow waves, high power density values in the delta frequency band (0.5 – 4.0 Hz) and lack of gross body movements. During REMS, the amplitude of the EEG is reduced, the predominant EEG power density occurs within the theta frequency (6.0 – 9.0 Hz) and there are phasic body twitches. During waking, the rats are generally active. There are protracted body movements. The amplitude of the EEG is similar to that observed during REMS, but power density values in the delta frequency band are generally greater than those in theta frequency band.

Experimental Protocol

The total of 35 Wistar rats were used in the postacquisition analysis and divided into 5 groups for this study. A 24-h undisturbed baseline EEG was recorded before initiating experiments in all groups. Rats in group 1 (n = 7) received PFS and 1 % ethanol 20 minutes prior to the beginning of the light (rest) period at the 1st and 2nd experimental days, respectively, and CBD 0.5 µg and 1.0 µg were randomly injected at the subsequent 3rd and 4th days. Rats which received a high dose on the 3rd day were given a low dose at the 4th day, and vice versa. The same protocol was employed after a two-day break in the same group of rats, except the injection was given at 20 minutes before the dark onset. Rats in group 2 (n = 7) were used to clarify the involvement of presynaptic 5-HT$_{1A}$ receptors by injections of buspirone (0.5, 2.0 or 4.0 µg) with 1.0 µg CBD 20 minutes prior to the light period. Seven rats in group 3 were administered ritanserin (5.0 or 10.0 µg) with 1.0 µg CBD 20 minutes prior to the light period to elucidate the effect of postsynaptic 5-HT$_2$ receptors on CBD-induced sleep alteration. Rats in group 4 (n = 8) were administered with 5-HT at doses of 3.0 and 10.0 µg to determine whether increase 5-HT concentration in the CeA exhibits the similar sleep alteration as that of CBD. These animals also received 4.0 µg buspirone or 10.0 µg ritanserin alone at 20 minutes before the light onset to demonstrate the effects of buspirone and ritanserin *per se* on sleep-wake activity during the light period. Rats in group 5 (n = 6) had a

similar protocol as those in group 2, except that those rats were received 100.0 or 300 pmol of ACEA with 1.0 µg CBD and were subsequently administered with 100.0 ng AM-251 alone. Rats in group 5 also received 300 pmol ACEA administration as a positive control and 10 % ethanol as a vehicle control after a two-day break. Sleep-wake activities were recorded at either the beginning of the light or the dark period after the injections and lasted for 24 hours.

Statistical Analyses

All values were presented as the mean ± SEM for the indicated sample sizes. Two-way (manipulation x time) re-
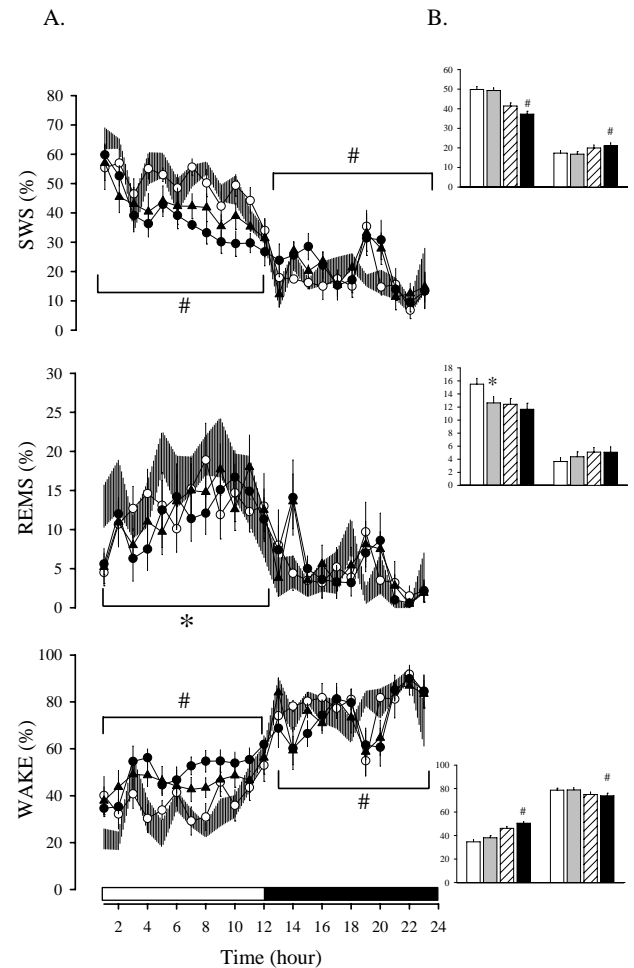

Fig. (2). The sleep-wake alteration induced by administration of CBD into CeA. **A**. Left panel: shaded area, open circles, closed triangles and closed circles represent the values obtained after injections of PFS, 1 % ethanol, 0.5 µg CBD and 1.0 µg CBD, respectively. The dark and open portions of horizontal bars represent the dark and light periods of the 12:12h light:dark cycle. WAKE, wakefulness; REMS, rapid eye-movement sleep; SWS, slow wave sleep. **B**. Right panel: the summary bar graph of each vigilance state. Left four bars represent the data acquired during the light period after administrations of PFS, 1 % ethanol, 0.5 µg CBD and 1.0 µg CBD, respectively, and the other four bars depict the values obtained in the subsequent 12-h time block (the dark period). * represents statistically significant difference between the values obtained from PFS and 1 % ethanol. # denotes the statistically significant difference between the values obtained from 1 % ethanol and those from CBD.

peated measures analyses of variance (ANOVA) for the duration of each vigilance state (SWS, REMS, WAKE) and for sleep architecture parameters were performed, comparing before and after manipulation within subjects, across the two 12-h time blocks. The comparison of latency of different vigilance states between manipulations has been done by one-way ANOVA. An α level of $\underline{p} \leq 0.05$ was taken as indicating a statistically significant difference.

RESULTS

Effects of CBD on Sleep-Wake Activity

All vigilance states (SWS, REMS, WAKE) were not altered after administering PFS when compared with the data acquired from the undisturbed baseline recordings (data not shown), which is consistent with our previous observation [26]. Since CBD was dissolved in 1 % ethanol, we determined the sleep-wake alteration after administering 2 µl of 1 % ethanol directly into the left CeA. Administration of 1 % ethanol 20 minutes prior to the beginning of the light period did not alter SWS during the 12-h light period when compared with those data acquired after administration of PFS. The total time spent in SWS after injection of PFS and 1% ethanol was 49.8 ± 1.5 % and 49.3 ± 1.4 % respectively (Fig. **2**). REMS, however, decreased from 15.5 ± 0.9 % obtained after PFS administration to 12.6 ± 1.0 % after given 1 % ethanol (n = 7; manipulation $F_{(1,6)} = 5.62$, p = 0.037; time $F_{(11,66)} = 3.32$, p = 0.117; Fig. **2**). The effects of ethanol on REMS may vary depending on the concentrations of ethanol, since there was no significant change of REMS when 10 % ethanol (the vehicle for ACEA) was administered into the rats in group 5. The percentages of REMS after administra-

tion of PFS and 10 % ethanol were 17.6 ± 1.0 % and 19.9 ± 1.1 % (manipulation $F_{(1,6)} = 3.60$, p = 0.106; time $F_{(11,66)} = 4.60$, p = 0.076), respectively. Microinjections of 0.5 and 1.0 µg CBD dose-dependently decreased SWS during the light period; the amounts of time spent in SWS during the light period were 41.4 ± 1.5 % (manipulation $F_{(1,6)} = 5.43$, p = 0.059; time $F_{(11,66)} = 3.34$, p = 0.118; when compared with the data obtained after 1 % ethanol) and 37.9 ± 1.5 % (manipulation $F_{(1,6)} = 26.35$, p = 0.002; time $F_{(11,66)} = 6.87$, p = 0.04; when compared with the data obtained after 1 % ethanol), respectively (Fig. **2**). REMS during the light period was not altered by CBD. There was a mirror effect of wakefulness enhancement after CBD administration (Fig. **2**). SWS during the subsequent 11-h dark period was significantly enhanced when 1.0 µg CBD was administered prior to the beginning of the light period (manipulation $F_{(1,6)} = 6.30$, p = 0.046; time $F_{(10,60)} = 3.77$, p = 0.10; when compared with the data obtained after 1 % ethanol; Fig. **2**). The enhancement of SWS during the subsequent dark period might be a compensatory effect due to the sleep debt in the previous light period, because our results demonstrated that CBD has no statistically significant effect on any aspect of sleep during the dark period when it was injected into CeA before the dark onset. The percentages of time spent in SWS and REMS during the 12-h dark period after administration of 1 % ethanol were 16.5 ± 1.3 % and 4.2 ± 0.7 %, and those obtained after injection of 1.0 µg CBD were 18.9 ± 1.4 % (manipulation $F_{(1,6)} = 2.59$, p = 0.159; time $F_{(11,66)} = 2.42$, p = 0.171) and 4.9 ± 0.7 % (manipulation $F_{(1,6)} = 0.24$, p = 0.644; time $F_{(11,66)} = 1.86$, p = 0.222).

Although the amount of REMS after receiving 1 % ethanol was decreased, analysis of sleep-architecture parameters

Table 1.   Effects of CBD, Buspirone, Ritanserin, ACEA and AM-251 on Sleep-Wake Architecture Parameters of Rats

Manipulation[4]	Hour	L:D Cycle[5]	Number of Bouts[1]			Bout Duration[2]			Transitions[3]
			WAKE[6]	SWS[6]	REMS[6]	WAKE	SWS	REMS	
PFS	1-12	L	6.0 ± 0.5	9.6 ± 0.4	4.0 ± 0.4	3.7 ± 0.4	3.2 ± 0.1	2.2 ± 0.2	49.9 ± 3.2
1 % EtOH	1-12	L	6.5 ± 0.5	10.0 ± 0.4	3.5 ± 0.6	3.7 ± 0.4	2.9 ± 0.2	1.9 ± 0.3	57.2 ± 2.5
CBD	1-12	L	$8.8 \pm 0.8^*$	11.0 ± 1.1	3.2 ± 0.5	3.8 ± 0.5	$1.9 \pm 0.1^*$	1.8 ± 0.3	$66.6 \pm 2.8^*$
CBD	1-12	L	7.5 ± 0.4	9.4 ± 0.6	2.9 ± 0.3	4.6 ± 0.7	2.0 ± 0.3	2.1 ± 0.2	63.9 ± 5.2
Buspirone + CBD	1-12	L	$6.1 \pm 0.6^\#$	9.2 ± 0.3	3.4 ± 0.6	3.8 ± 0.3	$3.1 \pm 0.3^\#$	1.9 ± 0.2	$52.4 \pm 2.2^\#$
CBD	1-12	L	7.4 ± 0.5	9.3 ± 0.7	2.9 ± 0.4	4.6 ± 0.8	2.1 ± 0.4	2.1 ± 0.2	63.3 ± 6.2
Ritanserin + CBD	1-12	L	$6.4 \pm 0.4^\#$	10.6 ± 0.7	4.0 ± 0.8	3.4 ± 0.5	$3.1 \pm 0.2^\#$	1.5 ± 0.3	$40.6 \pm 2.9^\#$
CBD	1-12	L	7.5 ± 0.3	10.1 ± 0.3	2.1 ± 0.6	3.9 ± 0.6	2.5 ± 0.1	2.3 ± 0.7	43.1 ± 3.1
ACEA +CBD	1-12	L	7.5 ± 0.3	10.5 ± 0.4	2.3 ± 0.5	3.1 ± 0.6	2.7 ± 0.1	2.7 ± 0.5	37.3 ± 3.1
PFS	1-12	L	5.1 ± 0.2	10.6 ± 0.3	4.7 ± 0.6	3.6 ± 0.4	2.9 ± 0.1	2.1 ± 0.1	43.1 ± 2.4
AM-251	1-12	L	$6.1 \pm 0.3^*$	$9.0 \pm 0.4^*$	$2.0 \pm 0.6^*$	$6.0 \pm 1.6^*$	2.9 ± 0.2	2.4 ± 0.7	$31.0 \pm 1.5^*$

Values are Means ± S.E.M. * denotes a statistically significant difference ($p < 0.05$) between values obtained after administration of vehicle (pyrogen-free saline [PFS] or 1% ethanol [EtOH]) and those obtained after receiving substances. # depicts a statistically significant difference ($p < 0.05$) between values obtained after CBD administration and those obtained after substance+CBD administration.
[1]Number of bouts per hour (mean ± SEM) for each vigilance state.
[2]Mean (± SEM) bout duration (min) for each vigilance state.
[3]Number of transitions from one behavioral state to another (mean ± SEM) per hour.
[4]Experimental manipulation: PFS = pyrogen-free saline (vehicle); EtOH = ethanol (vehicle).
[5]Period of the light:dark cycle immediately prior to which injections were given: L = light period.
[6]Vigilance states: WAKE, wakefulness; SWS, slow-wave sleep; REMS, rapid eye movements sleep.

across hours 1 to 12 revealed that both the number of REMS bouts and the REMS bout duration were not significantly altered (Table **1**). The reduction in SWS and increase of wakefulness during the light period induced by CBD was primarily due to a decrease in SWS bout duration and an enhancement in the number of wakefulness bouts when compared to those parameters obtained after injection of 1 % ethanol (Table **1**). The number of transition from one state of vigilance to another during the 12-h light period increased significantly after CBD administration, indicative of sleep fragmentation (Table **1**). EEG slow wave activities (SWAs) during SWS were not altered; SWAs during SWS in the 12-h light period were 912.4 ± 0.5 and 912.2 ± 0.5 $\mu V^2/Hz$ obtained after 1 % ethanol and 1.0 μg CBD, respectively. Administration of 1 % ethanol or 1.0 μg CBD did not significantly alter the latencies for entering the states of SWS and REMS from the beginning of the light period. The SWS latencies after administrations of PFS, 1 % ethanol and 1.0 μg CBD were 1.9 ± 1.4, 13.5 ± 6.1 (n = 7, p = 0.185 when compared to PFS, one-way ANOVA) and 5.3 ± 3.8 minutes (p = 0.860 when compared to PFS, one-way ANOVA), and the REMS latencies were 23.7 ± 10.9, 49.1 ± 11.2 (p = 0.324 when compared to PFS, one-way ANOVA) and 40.5 ± 12.6 minutes (p = 0.599 when compared to PFS, one-way ANOVA), respectively.

The Involvement of Serotonergic Neurons in CBD-Induced Sleep Alteration

Administrations of 5-HT$_{1A}$ receptor agonist, buspirone, dose-dependently blocked CBD-induced SWS decrease. The total amount spent in SWS acquired after double injections of 0.5, 2.0 or 4.0 μg buspirone with CBD increased from 37.4 ± 3.2 % obtained after receiving 1.0 μg CBD to 40.2 ± 1.6 % (manipulation $F_{(1,6)} = 0.53$, p = 0.494; time $F_{(11,66)} = 5.27$, p = 0.061), 43.0 ± 1.6 % (manipulation $F_{(1,6)} = 2.10$, p = 0.198; time $F_{(11,66)} = 5.28$, p = 0.061) or 49.2 ± 1.5 % (manipulation $F_{(1,6)} = 17.72$, p = 0.006; time $F_{(11,66)} = 7.52$, p = 0.034), respectively (Fig. **3**). The CBD-induced suppressive effect in REMS is due to the non-specific effect of 1 % ethanol vehicle as previous described. Buspirone exhibited no further effect on REMS in this group of rats (Fig. **3**). Furthermore, our results have demonstrated that single administration of a high dose of buspirone (4.0 μg) prior to the beginning of the light period did not significantly alter both SWS and REMS during the light period (Fig. **5A**). Administration of ritanserin also dose-dependently blocked CBD's effect on SWS during the light period. The percentages of time spent in SWS increased from 37.5 ± 1.5 % after receiving CBD to 47.9 ± 1.7 % (manipulation $F_{(1,6)} = 8.00$, p = 0.030; time $F_{(11,66)} = 3.42$, p = 0.114) and 51.6 ± 1.5 % (manipulation $F_{(1,6)} = 12.04$, p = 0.013; time $F_{(11,66)} = 4.59$, p = 0.076) obtained after 5.0 μg ritanserin+CBD and 10.0 μg ritanserin+CBD, respectively (Fig. **4**). Ritanserin also exhibited no further effect on REMS in this group of rats (Fig. **4**). A high dose of ritanserin, 10.0 μg, administered before the beginning of the light period did not significantly alter both SWS and REMS (n = 7, Fig. **5A**). Furthermore, administration of serotonin at doses of 3.0 and 10.0 μg prior to the beginning of the light period significantly suppressed SWS, which is similar to the effect of CBD on SWS. The total amounts of time spent in SWS were decreased from 49.2 ± 1.2 % after receiving PFS injection to 41.8 ± 1.4 % (manipu-

lation $F_{(1,6)} = 6.29$, p = 0.046; time $F_{(11,66)} = 2.37$, p = 0.174) and 41.2 ± 1.3 % (manipulation $F_{(1,7)} = 8.54$, p = 0.022; time $F_{(11,77)} = 3.50$, p = 0.103) obtained after 3.0 and 10.0 μg serotonin administrations, respectively (Fig. **5B**). In addition, serotonin also reduced REMS during the light period, which is not exhibited by CBD administration. The percentages of REMS decreased from 18.6 ± 0.9 % after receiving PFS injection to 13.7 ± 0.9 % (manipulation $F_{(1,6)} = 7.77$, p = 0.032; time $F_{(11,66)} = 7.73$, p = 0.032) and 13.5 ± 0.9 % (manipulation $F_{(1,7)} = 10.10$, p = 0.016; time $F_{(11,77)} = 7.75$, p = 0.027) obtained after 3.0 and 10.0 μg serotonin administrations, respectively (Fig. **5B**).
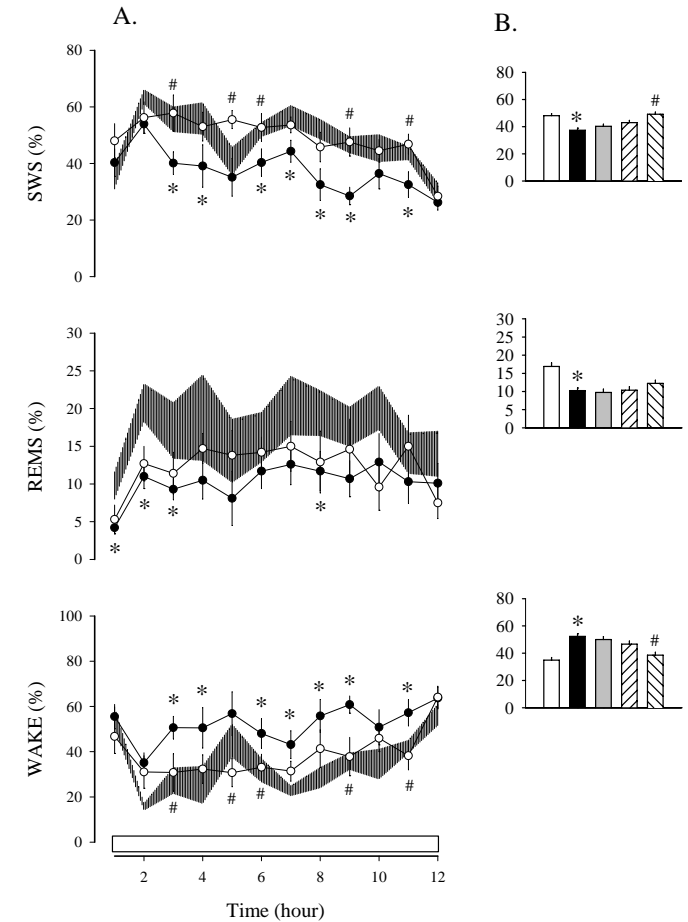

Fig. (3). Effects of presynaptic 5-HT$_{1A}$ receptor agonist, buspirone on CBD-induced sleep alteration. **A**. Left panel: shaded area, closed circles and open circles represent the values obtained after injections of PFS, 1.0 μg CBD and 4.0 μg buspirone+1.0 μg CBD, respectively, during the light period. **B**. Right panel: the summary bar graph of each vigilance state. The bars from left to right represent the data acquired during the 12-h light period after administrations of PFS, 1.0 μg CBD, 0.5 μg buspirone+1.0 μg CBD, 2.0 μg buspirone+1.0 μg CBD and 4.0 μg buspirone+1.0 μg CBD, respectively. * represents statistically significant difference between the values obtained from PFS and CBD. # denotes the statistically significant difference between the values obtained from buspirone+CBD and those from CBD.
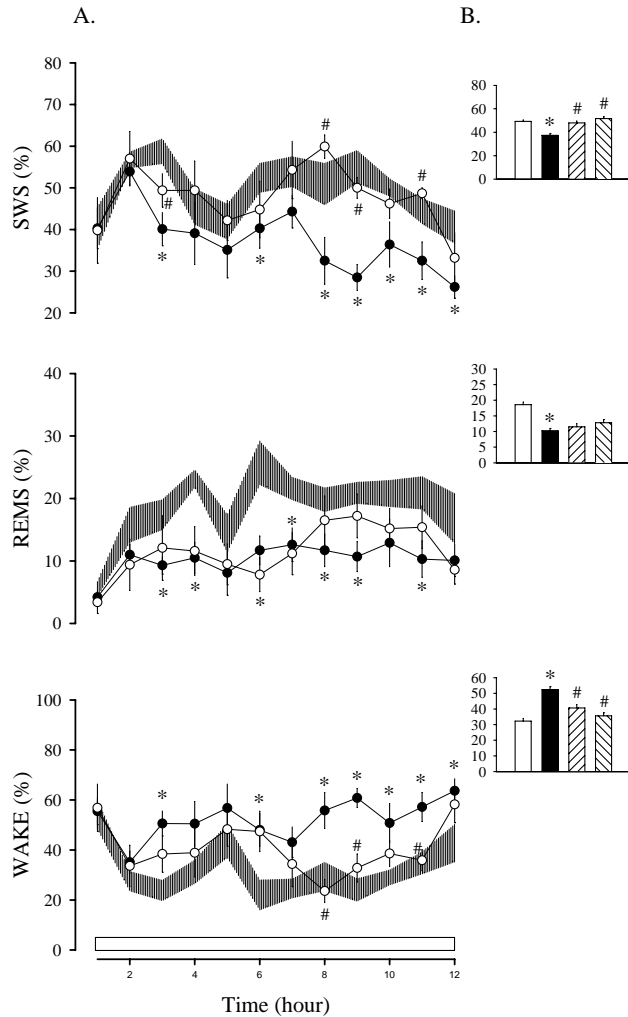
sitions when comparing to that obtained after CBD, suggesting that buspirone improves CBD-induced sleep fragmentation (Table **1**). Application of ritanserin has similar effect as that of buspirone on reversing CBD-induced alteration in sleep-architecture parameters (Table **1**).

The Effects of CB₁ Receptor Agonist (ACEA) and Antagonist (AM-251)

Administration of 300 pmol ACEA alone into the CeA exhibited no significant effect on all vigilance states during the 12-h light period when compared with the data acquired after administration of 10 % ethanol (the vehicle). The amounts of time spent in SWS obtained after administrations of 10 % ethanol and ACEA were 50.3 ± 1.8 % and 51.6 ± 2.3 %, respectively (manipulation $F_{(1,5)} = 4.275$, p = 0.094; time $F_{(11,55)} = 3.615$, p = 0.116). The amounts of REMS were 20.6 ± 2.0 % and 18.9 ± 1.1 % obtained after 10 % ethanol and ACEA, respectively (manipulation $F_{(1,5)} = 3.226$, p = 0.132; time $F_{(11,55)} = 5.491$, p = 0.066). Microinjections of CB₁ agonist ACEA into the CeA partially and dose-dependently reversed the CBD-induced SWS decrease during the light period, although it did not reach statistical significance during the 12-h time block. Administration of 300 pmol ACEA increased the total amount of time spent in SWS during the light period from 42.9 ± 1.5 % obtained after administration of 1.0 µg CBD to 48.0 ± 1.7 % (manipulation $F_{(1,5)} = 2.83$, p = 0.168; time $F_{(11,55)} = 1.44$, p = 0.297; Fig. **6**). However, ACEA did significantly block CBD-induced SWS decrease at several time points, such as hours 5, 8 and 11 (Fig. **6**). Our result also demonstrated that micro-administration of CB₁ receptor antagonist AM-251, 100.0 ng exhibits a similar effect as that of CBD on sleep-wake activity, except its efficacy is less than CBD's. AM-251 reduced SWS and enhanced wakefulness, but did not alter REMS. The percentages of SWS obtained after administering PFS (vehicle for AM-251), 1 % ethanol (vehicle for CBD), CBD and AM-251 were 52.7 ± 1.5 %, 51.6 ± 1.7 %, 42.9 ± 1.5 % (manipulation $F_{(1,5)} = 18.78$, p = 0.007; time $F_{(11,55)} = 1.16$, p = 0.330; when compared with data obtained from 1 % ethanol) and 45.1 ± 2.8 % (manipulation $F_{(1,5)} = 9.22$, p = 0.029; time $F_{(11,55)} = 2.32$, p = 0.189; when compared with data obtained from PFS); the amounts of REMS were 18.9 ± 1.5 %, 13.2 ± 1.2 %, 12.2 ± 1.3 % and 11.2 ± 3.6 %, respectively.

Analysis of sleep-architecture parameters across hours 1-12 during the light period revealed that ACEA+CBD did not alter any aspect of sleep-wake architecture parameters when comparing with those obtained after CBD administration (Table **1**), although ACEA partially blocked CBD-induced SWS decrease. Although AM-251 has a similar effect on SWS as CBD does, the alteration in sleep architecture is different. The reduction in SWS and enhancement of wakefulness induced by AM-251 were primarily due to the reduction in SWS bout's numbers and the increases of both the wakefulness bout's numbers and duration. The transition from one state of vigilance to another was decreased, which differs from the effect of CBD (Table **1**).

DISCUSSION

The amygdala appears to be a critical brain structure which involves in the emotional, behavioral, and physiological responses associated with fear and anxiety [27-29]. In addition, the role of amygdala on arousal and sleep regula-
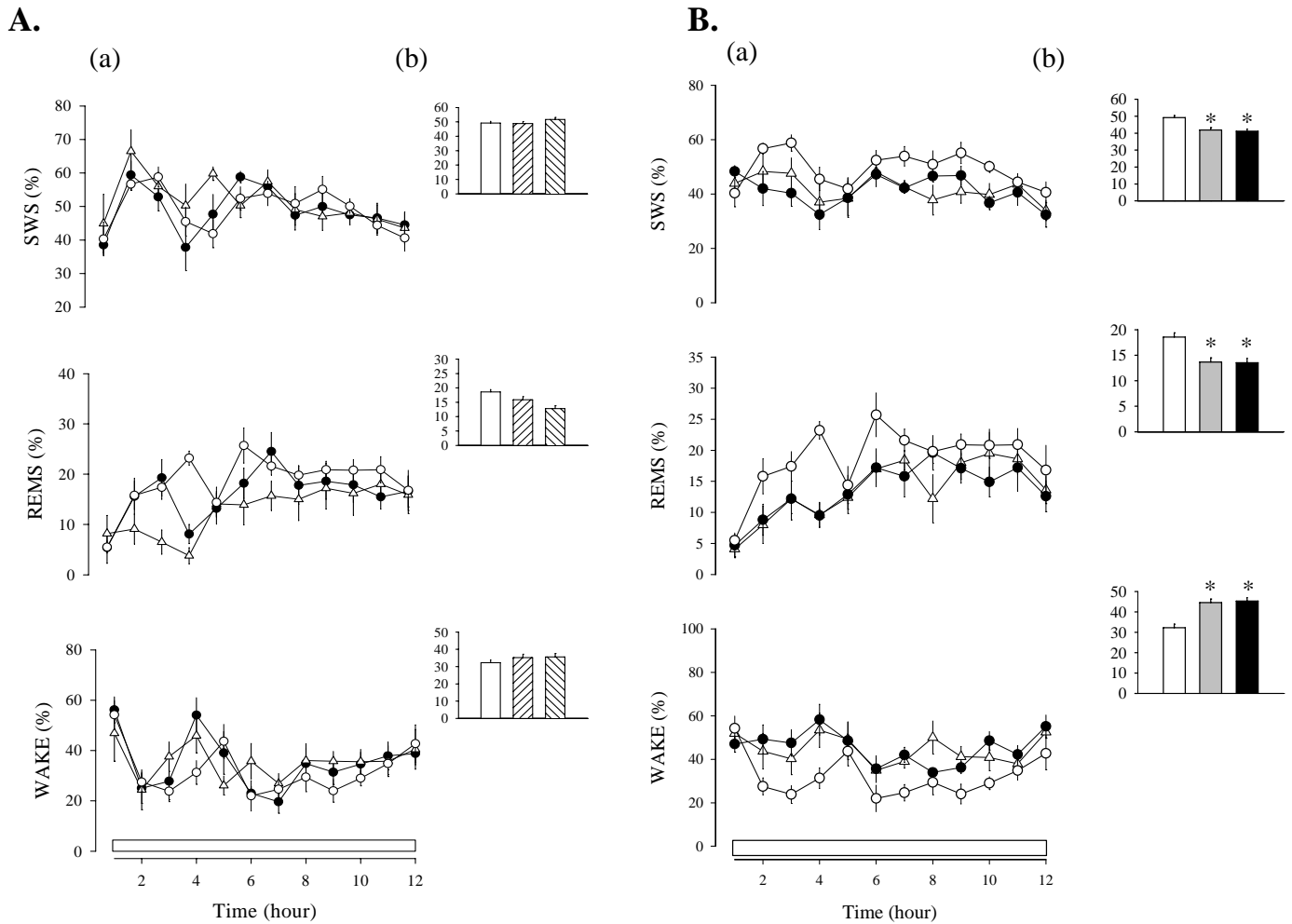

Fig. (4). Effects of postsynaptic 5-HT₂ receptor antagonist, ritanserin on CBD-induced sleep alteration. **A**. Left panel: shaded area, closed circles and open circles represent the values obtained after injections of PFS, 1.0 µg CBD and 10.0 µg ritanserin+1.0 µg CBD, respectively, during the light period. **B**. Right panel: the summary bar graph of each vigilance state. The bars from left to right represent the data acquired during the 12-h light period after administrations of PFS, 1.0 µg CBD, 5.0 µg ritanserin+1.0 µg CBD and 10.0 µg ritanserin+1.0 µg CBD, respectively. * represents statistically significant difference between the values obtained from PFS and CBD. # denotes the statistically significant difference between the values obtained from ritanserin+CBD and those from CBD.

The blockade of CBD-induced decrease in SWS and increase in wakefulness by buspirone was primarily due to an increase in SWS bout duration and a decrease in number of wakefulness bouts (Table **1**). This observation indicates that buspirone reverses CBD-induced alteration of sleep-architecture parameters during the light period. In addition, buspirone+CBD significantly decreased the number of tran-

A.

B.

Fig. (5). A: The effects of buspirone and ritanserin on spontaneous sleep during the light period. (a). Left panel: open circles, closed circles and open triangles represent the values obtained after injections of PFS, 4.0 µg buspirone and 10.0 µg ritanserin, respectively, during the light period. (b). Right panel: the summary bar graph of each vigilance state. The bars from left to right represent the data acquired during the 12-h light period after administrations of PFS, 4.0 µg buspirone and 10.0 µg ritanserin, respectively. **B**: Sleep alteration induced by serotonin. (a). Left panel: open circles, open triangles and close circles represent the values obtained after injections of PFS, 3.0 µg serotonin and 10.0 µg serotonin, respectively, during the light period. (b). Right panel: the summary bar graph of each vigilance state. The bars from left to right represent the data acquired during the 12-h light period after administrations of PFS, 3.0 µg serotonin and 10.0 µg serotonin, respectively. * represents statistically significant difference between the values obtained from PFS and serotonin.

tion has also been emphasized [12,30,31]. Electrical stimulation of CeA suppresses delta wave activity in the frontal cortex and increases neocortical arousal [30]. Tang *et al.* have demonstrated that inactivating CeA neurons with tetrodotoxin (TTX), a sodium channel blocker, decreases REMS and increases SWS [31]. Administration of GABA$_A$ receptor agonist, muscimol, into the CeA suppresses REMS, but has no effect on SWS [12]. Lesion of amygdala produces more sleep and high proportion of REMS in rhesus monkeys [32]. Furthermore, neurons in the CeA send their outputs to various target structures, including the ventral tegmental area, locus coeruleus, lateral dorsal tegmental nucleus and basal forebrain, which are implicated in the behavioral and EEG arousal, the increased vigilance and the augmented attention [28]. As for the input innervations, it has been demonstrated that the rat's amygdala receives highly dense projections of serotonergic fibers from the dorsal raphe nucleus [33,34]. Moreover, sub-nuclei of macaque amygdala, such as the central nucleus, nucleus of the lateral olfactory tract, paralami-nar nucleus and anterior amygdaloid area, are densely innervated by serotonergic fibers [16]. Serotonergic receptors, especially the 5-HT$_1$ and 5-HT$_2$ receptors, are widely distributed throughout the amygdala [35,36]. The serotonergic system has been implicated in the mechanisms of fear and anxiety. For example, the selective serotonin uptake inhibitor (SSRI) is one of the efficacious medications in treating anxiety [37]. The genetic variation of the 5-HT transporter is correlated with the anxious behavior in humans and primates [38,39]. In addition to participate in fear and anxiety, serotonin has been widely proposed as a waking promoter. Evidence has demonstrated that systemic administration of 5-HT$_{1A}$ receptor selective agonist increases wakefulness and sleep latency, and reduces REMS [40]. Blockade of 5-HT$_{2A}$ receptors results in an increase of NREMS in the 5-HT$_{2A}$ +/+ (wild-type) mice, but not in the 5-HT$_{2A}$ -/- (knock-out) mice [41]. The discharging rate of major cells in the CeA is higher during wakefulness and REMS rather than during SWS [42]. Microinjection of 5-HT antagonist into the amygdala in-
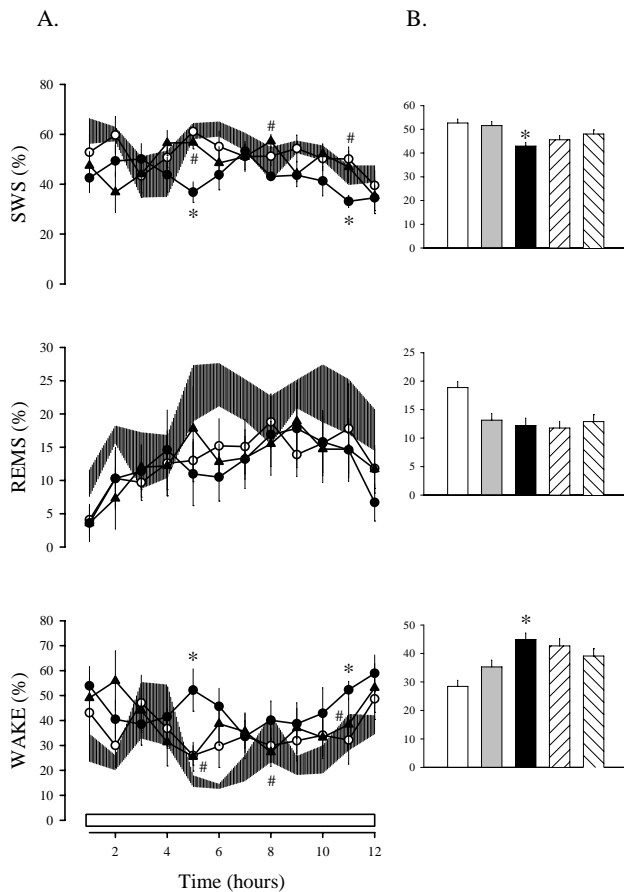
Fig. (6). The effects of CB1 receptor agonist, ACEA on CBD-induced sleep alteration. **A**. Left panel: shaded area, open circles, closed circles and closed triangles represent the values obtained after injections of PFS, ethanol, 1.0 μg CBD and 300 pmol ACEA+1.0 μg CBD, respectively, during the light period. **B**. Right panel: the summary bar graph of each vigilance state. The bars from left to right represent the data acquired during the 12-h light period after administrations of PFS, ethanol, 1.0 μg CBD, 100 pmol ACEA+1.0 μg CBD and 300 pmol ACEA+1.0 μg CBD, respectively. * represents statistically significant difference between the values obtained from ethanol vehicle and CBD. # denotes the statistically significant difference between the values obtained from ACEA+CBD and those from CBD.

creases sleep efficiency and ponto-geniculo-occipital (PGO) wave frequency in wakefulness and SWS [13]. Aforementioned observations suggest the involvement of amygdaloid serotonin in the sleep-waking regulation.

Our current observations are the first to demonstrate the sleep-wake alteration induced by a direct administration of CBD into the CeA and to elucidate its possible underlying mechanisms. CBD is the first compound isolated from the cannabis plant and it exhibits a broad pharmacological profile, including anti-convulsion, sedation, anti-anxiety, hypnotic effect, anti-psychosis and anti-inflammation [11].

Those pharmacological properties are more predominant in the therapeutic purposes rather than other active compounds, such as THC, because it is non-psychoactive [5]. Currently a cannabis extract, Sativex®, which comprises THC and CBD in a ratio of 1:1, is used clinically. It has been reported that Sativex® dramatically improves subjective sleep parameters in patients with a variety of pain [43]. Orally taking CBD-predominant extract causes a mild activation effect, whereas taking THC results in a slight residual sedation [43]. Monti has indicated that intraperitoneal injections of CBD in a high and a low doses into rats exhibit an opposite effect on SWS latency [21]. Furthermore, ICV administration of CBD increases waking and decreases REMS in rats during the light period [22]. However, the underlying mechanism of CBD on sleep-wake activity remains unclear. Our result elicited that no any aspect of sleep-wake parameters during the dark period was altered when CBD was directly administered in to the CeA prior to the dark onset. However CBD produced a dose-dependent decrease of SWS during the light period when CBD was injected prior to the beginning of the light period. Our results also demonstrated that REMS was suppressed by 1 % ethanol (the vehicle for CBD), but not altered by 10 % ethanol (the vehicle for ACEA), suggesting that the effect of ethanol on sleep-wake activity may vary depending on the concentrations of ethanol. This observation is consistent with a previous result depicted by Ghosh *et al.* (1991) that ethanol inhalation decreases REMS at a lower dose, but exhibits no sleep alteration while a high concentration was given [44]. We then determined whether the CBD-induced sleep alteration is mediated by the activation of serotonergic system in the CeA, as we described previously that the CeA receives highly dense serotonergic projections from the raphe nucleus [33,34]. Evidence of a microdialysis study in which ICV administration of CBD into a rat increases the serotonin concentration in the nucleus accumbens at hours 1 and 2 after injection directs the possibility of serotonergic involvement [22]. Buspirone, which exhibits the anxiolytic effect through agonizing the presynaptic 5-HT$_{1A}$ receptors [45], was the first substance to elucidate the involvement of serotonin in the CBD-induced sleep alteration. Our result has shown that buspirone blocked CBD-induced SWS alteration in a dose-dependent manner, suggesting the CBD's effect on sleep alteration may be mediated by increasing serotonin activation/release from the presynaptic terminals of 5-HT fibers (Fig. **7**). This hypothesis is further confirmed by the blockade of CBD-induced SWS alteration by ritanserin, the post-synaptic 5-HT$_2$ receptor antagonist [14]. In addition to the alteration of total amounts of sleep, analyses of sleep-architecture parameters elicited that both buspirone and ritanserin exhibit the same direction to block the reduction of SWS bout's duration and the enhancement of waking bout's numbers produced after CBD administration. Administration of serotonin into the CeA mimicked CBD-induced SWS reduction, but it also reduced REMS. The amygdaloid serotonin has been implicated in REMS regulation in addition to the SWS. For example, REM-ON neurons recorded from the CeA were inhibited when stimulation of neurons in the dorsal raphe nucleus [42]. In addition, it has been reported that increasing activity of neurons in the locus coeruleus and raphe nucleus suppresses the activity of cholinergic pedunclopontine REM sleep effector cells [46]. Nevertheless, CBD has no further effect on REMS during the light period, which
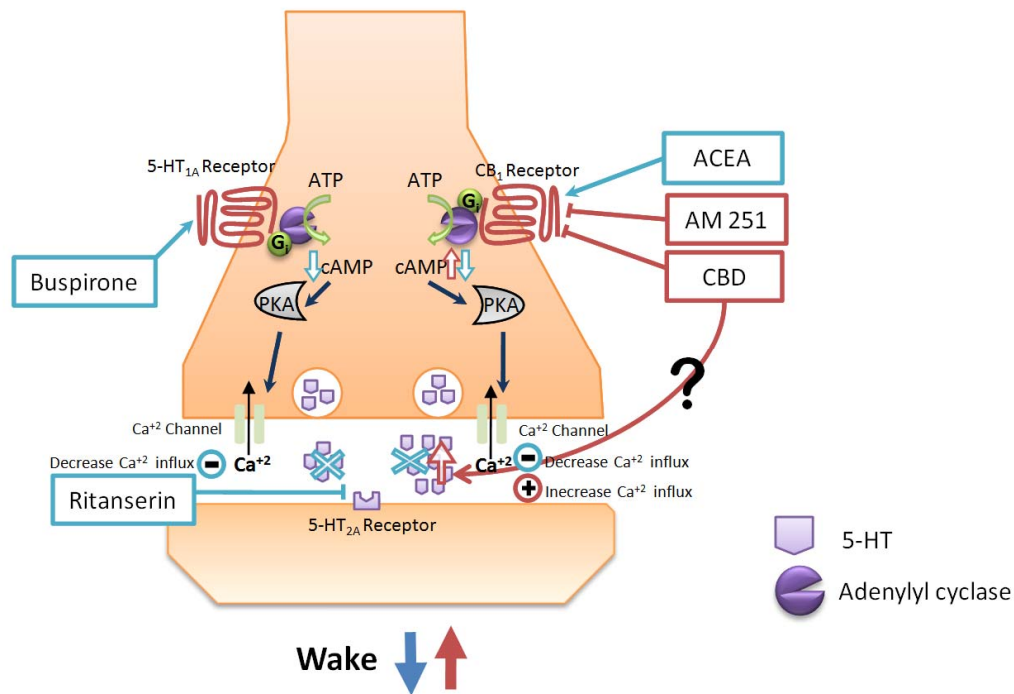
Fig. (7). The scheme of CBD's mechanism. CBD-induced SWS decrease is partially mediated by antagonizing presynaptic CB_1 receptors at the serotonergic terminals, which results in the cAMP enhancement, and subsequently enhancing serotonin release from axon terminals. Additionally, there is an unidentified mechanism (indicated as "?") which may also contribute to the CBD's action. "⊥" indicates the antagonizing action; "←" depicts the agonizing effect; blue color indicates the pathway for decreasing wakefulness; red color represents the pathway for increasing waking. PKA: protein kinase A.

might be due to that ethanol (vehicle) masks CBD's effect and/or CBD is less effective to activate the serotonergic system in amygdala than an exogenous 5-HT injection does.

The function of endocannabinoid system has been demonstrated that the endocannabinoid produced from postsynaptic neurons serves as a retrograde signaling and binds to CB_1 receptors on presynaptic terminals to inhibit neurotransmitter release [4,17]. The presynaptic CB_1 receptors located on axon terminals are highly expressed in the basal ganglia, cerebellum, hippocampus and olfactory cortex, and are moderately expressed in the cerebral cortex, amygdala, septum and brain stem [6-8]. Evidence suggests that cannabinoid CB_1 receptors and 5-HT transporters are co-localized in the amygdala [18], implying that activation of presynaptic cannabinoid CB_1 receptors might reduce 5-HT release from axon terminals. It has been further demonstrated that Δ^9-THC-induced catalepsy-like immobilization is mediated by decreased 5-HT neurotransmission in the nucleus accumbens [47], and that CB_1 receptor protein exists on serotonergic fibers and synapses expressing the 5-HT uptake transporter in both the hippocampus and amygdala [48]. Although evidence suggests that CBD has a low binding affinity to the CB_1 receptors [5,11], it has recently been emphasized that CBD displays a high potency on antagonizing CB_1 receptors in the mouse vas deferens [23] and in mouse brain [24]. They found that CBD antagonizes both CP 55940-induced and R-(+)-WIN 55212-mediated stimulations of [^{35}S]GTPγS binding to the mouse brain, which demonstrates the similar effects of CB_1 receptor antagonists, rimonabant and O-2654 [24]. The K_B value of CBD for antagonizing CP 55940-induced stimulation of [^{35}S]GTPγS binding to the

mouse brain was below its corresponding CB_1 K_i value [24]. Both CP 55940 and R-(+)-WIN 55212 are the cannabinoid receptor agonists. We further elucidated the involvement of presynaptic CB_1 receptors by employing a CB_1 agonist ACEA, and found that ACEA partially and dose-dependently reversed the CBD-induced SWS decrease during the light period. Furthermore, a CB_1 specific antagonist, AM-251, produced a similar reduction in SWS during the light period, although the effect of AM-251 was less effective than that of CBD. Analyses of sleep-wake architecture parameters revealed that AM-251 increased the bout duration of wakefulness and decreased the numbers of SWS and REMS bouts in addition to the increase of waking bout's numbers that was also produced by CBD. However, CBD-induced decrease of SWS bout's duration was not observed by AM-251. The observation implicates that antagonizing presynaptic CB_1 receptor on serotonergic fibers only partially explain the actions of CBD on sleep alteration, additional mechanism(s) may involve in its sleep regulation. In fact, it has been proposed that the effects of CBD at anti-convulsion, anti-anxiety, anti-psychosis, anti-nausea and anti-rheumatoid arthritic properties might be because of the inhibition of endogenous endocannabinoid (anandamide) uptake and hydrolysis or the anti-oxidative effect [11,49]. If the inhibition of anandamide hydrolysis is involved in CBD-induced sleep alteration, the increased anandamide would decrease 5-HT release *via* an agonistic action of presynaptic CB_1 receptors, which may result in an increase of SWS. However, this conjectural outcome conflicts with our current observation of SWS decrease caused by CBD. Nerverthless, investigating further possible mechanism(s) that contributes to the CBD's

sleep regulation is worthwhile, since the underlying mechanisms of CBD are complicated and remain ambiguous. In addition, the amygdaloid CBD-induced sleep alteration is unexpected and paradoxical. In clinical, sleep disruption is commonly found in patients with anxiety, such as the generalized anxiety disorder, panic disorder, and posttraumatic stress disorder [50]. Conversely, sleep deprivation exacerbates the relapse of anxiety condition [50]. Anxiogenic agents mostly produce arousal activation and anxiolytics conversely reduce arousal. Nevertheless, few anxiolytics exhibit the CBD-like paradoxical effect of arousal stimulatation. For instance, nicotine increases the inhibition of sleep-promoting systems facilitated by noradrenaline [51], and enhances discharge rate of dorsal raphe 5-HT neurons during REMS [52]. Another example is that neuropeptide S exerts both anxiolytic and arousal effects in rodents [53]. Our results demonstrated that CBD exerts an unique pharmacological property to promote the wakefulness in addition to its well-known anxiolytic effect. Furthermore, it also suggests that two distinct mechanisms may dominate the different actions of CBD, the anti-anxiety and sleep-wake regulation. Interfering hypothalamic neuropeptides, such as corticotropin-releaseing hormone (CRH) that is well known to involve in the stress response, may play a role in CBD's anxiolytic properties. However, the anxiolytic mechanism of CBD and its role of sleep-wake regulation in an anxious rat need to be further investigated.

CONCLUSION

In conclusion, our current results demonstrated the sleep regulation of amygdaloid CBD as the scheme depicting in Fig. (**7**). CBD antagonizes CB_1 receptors at the pre-synaptic terminals of serotonergic fibers in the CeA, enhances the release of serotonin, and subsequently binds to the post-synaptic $5-HT_2$ receptors to reduce SWS and increase wakefulness. Serotonin and CB_1 receptor antagonist AM-251 could produce a similar effect as that CBD does, and the CBD-induced SWS reduction was blocked by CB_1 agonist, presynaptic $5-HT_{1A}$ agonist and post-synaptic $5-HT_2$ antagonist. These results elicit the possible mechanism of CBD on sleep-wake regulation and provide a therapeutic direction for CBD in treating somnolence.

DISCLOSURE/CONFLICT OF INTEREST

This is not an industry supported study. Authors have indicated no financial conflicts of interest.

ACKNOWLEDGEMENTS

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