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Professor Verpoorte

Gorlaeus Laboratorium, HB024, Universiteit Leiden, Einsteinweg 55, 2333 CC Leiden, Netherlands

September 28, 2010

Dear Professor Verpoorte:

Please find enclosed a manuscript, entitled "**Biphasic effects of baicalin, an active constituent of *Scutellaria baicalensis* Georgi, in the spontaneous sleep-wake regulation**", for consideration for publication in *Journal of Ethnopharmacology*. The data contained in this manuscript will not be submitted for publication elsewhere until a decision has been made regarding its suitability for publication in *Journal of Ethnopharmacology*. This manuscript is in accordance with the Authorship statement of ethical standards for manuscripts submitted to *Journal of Ethnopharmacology*.

We online submitted the manuscript with six figures and one table. We trust you will find this manuscript suitable for publication in *Journal of Ethnopharmacology*, and look forward to hearing from you in the near future in this regard.

Sincerely,

Fang-Chia Chang, Ph.D.
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We thank the referees for finding our work of interest and for agreeing with us that this work is a valuable contribution to the field. The referees spent considerable effort in the review of this manuscript, and we thank them their comments and suggestions. We have incorporated many of suggestions into this revised version of the manuscript. We provide a detailed list of our responses here:

Responses to reviewer:

(1). The format of references has been changed according to the JEP format.

(2). The experimental details and interpretation in the figure legends have been removed. Readers could find those descriptions in the sections of Methods and Discussion.

(3). We added the abbreviations of the figures in the figure legends to avoid the complexity of the figures.

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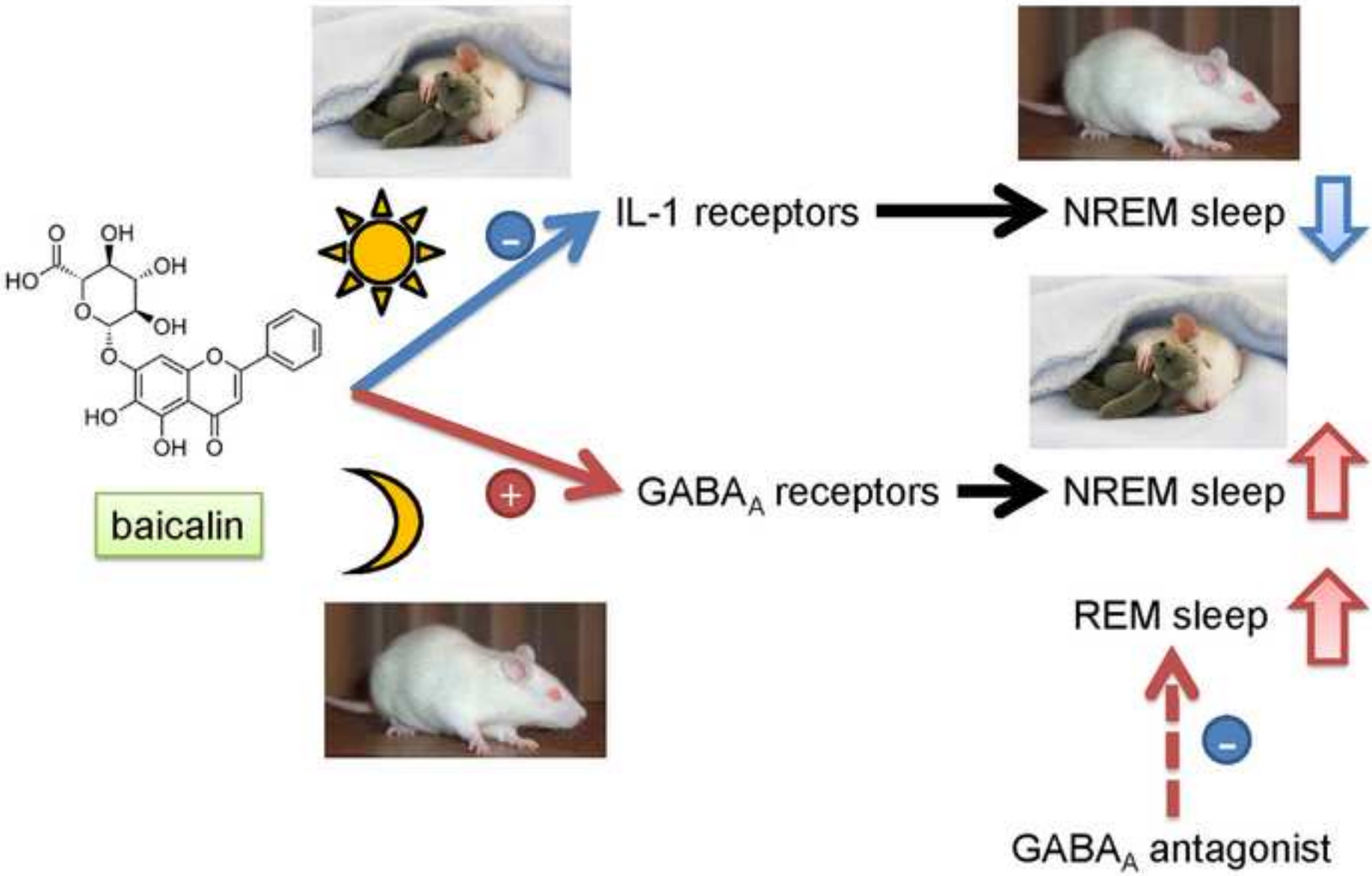

ABSTRACT

Aim of the study: Baicalin is an active compound originating from the root of *Scutellaria baicalensis* Georgi, which has been used for anti-inflammation, anti-bacteria, anti-hypertension, anti-allergy and sedation since ancient China, though the neuronal mechanisms involved in the sedative effect is still unclear. Baicalin possesses the ability to decrease the expression of pro-inflammatory cytokines and nuclear factor (NF)-κB activity. Furthermore, baicalin has demonstrated an anxiolytic-like effect via activation of γ-aminobutyric acid-A ($GABA_A$) receptors. Pro-inflammatory cytokines (e.g. interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α) and the GABAergic system promote sleep. This study was designed to determine whether the $GABA_A$ receptor activation and/or the suppression of pro-inflammatory cytokines mediate(s) baicalin-induced sleep alterations.

Materials and methods: Baicalin was intracerebroventricularly (ICV) administered 20 minutes either prior to the beginning of the light period or before the onset of the dark period. Electroencephalogram (EEG) and gross body movement were acquired for sleep analysis. Pharmacological blockade of IL-1 and $GABA_A$ receptors were employed to elucidate the involvements of IL-1 and $GABA_A$ receptors in baicalin-induced sleep alterations. IL-1β concentrations obtained after baicalin administration in several distinct brain regions were determined by ELISA.

Results: ICV administration of baicalin decreased slow wave sleep (SWS) during the first two hours of the light period. Rapid eye movement sleep (REMS) was not altered. The blockade of

IL-1β-induced SWS enhancement by baicalin suggests that the antagonism of IL-1 receptors is involved in baicalin-induced SWS decrement during the light period. However, IL-1β concentrations during the light period were not altered after baicalin administration. In contrast, baicalin increased both SWS and REMS during hours 8~10 of the dark (active) period when baicalin was administered at the beginning of the dark period, and its effects were blocked by the $GABA_A$ receptor antagonist bicuculline.

Conclusion: Baicalin exhibits biphasic effects on sleep-wake regulation; the decrease of SWS during the light period and increases of SWS and REMS during the dark period. Inhibition of IL-1 action and enhancement of $GABA_A$ receptor activity may mediate baicalin's effects during the light and dark period, respectively.

Biphasic effects of baicalin, an active constituent of *Scutellaria baicalensis* Georgi, in the spontaneous sleep-wake regulation

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Running title: Baicalin regulates spontaneous sleep

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ABSTRACT

Aim of the study: Baicalin is an active compound originating from the root of *Scutellaria baicalensis* Georgi, which has been used for anti-inflammation, anti-bacteria, anti-hypertension, anti-allergy and sedation since ancient China, though the neuronal mechanisms involved in the sedative effect is still unclear. Baicalin possesses the ability to decrease the expression of pro-inflammatory cytokines and nuclear factor (NF)-κB activity. Furthermore, baicalin has demonstrated an anxiolytic-like effect via activation of γ-aminobutyric acid-A ($GABA_A$) receptors. Pro-inflammatory cytokines (e.g. interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α) and the GABAergic system promote sleep. This study was designed to determine whether the $GABA_A$ receptor activation and/or the suppression of pro-inflammatory cytokines mediate(s) baicalin-induced sleep alterations.

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Results: ICV administration of baicalin decreased slow wave sleep (SWS) during the first two hours of the light period. Rapid eye movement sleep (REMS) was not altered. The blockade of IL-1β-induced SWS enhancement by baicalin suggests that the antagonism of IL-1 receptors is involved in baicalin-induced SWS decrement during the light period. However, IL-1β concentrations during the light

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Conclusion: Baicalin exhibits biphasic effects on sleep-wake regulation; the decrease of SWS during the light period and increases of SWS and REMS during the dark period. Inhibition of IL-1 action and enhancement of $GABA_A$ receptor activity may mediate baicalin's effects during the light and dark period, respectively.

Key words: Baicalin, *Scutellaria baicalensis* Georgi, IL-1, GABA, slow wave sleep (SWS)

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1. INTRODUCTION

Traditional Chinese medicinal herb *Scutellariae* Radix (Huang Qin), the dried root of *Scutellaria baicalensis* Georgi, has been documented to possess therapeutic effects, such as anti-inflammation, anti-pyretic, anti-bacteria, anti-hypertension, anti-allergy and sedation, and has been widely used for thousands of years in China and other Asian countries. However, the application of *Scutellaria baicalensis* in regulating the function of the central nervous system (CNS), such as sedation, is limited in clinical practice. Several researches have recently demonstrated the affirmed influences of *Scutellariae* Radix on the CNS. For example, intraperitoneal (i.p.) administration of methanol extracts from *Scutellaria baicalensis* significantly protects hippocampal CA1 neurons from transient ischemia-induced cell death (Kim et al., 2001). Transient cerebral ischemia-induced impairment of learning and memory in mice is spared after receiving a prescription of oren-gedoku-to (Huang-Lian-Jie-Du-Tang), in which *Scutellariae* Radix is the main ingredient (Xu et al., 2000). Baicalin (7-glucuronic acid-5,6-dihydroxyflavone) is a flavonoid compound purified from the *Scutellaria baicalensis*, which has been reported to possess several pharmacological attributes, including anti-inflammation (Krakauer et al., 2001; Chou et al., 2003), anti-viral activity (Kitamura et al., 1998; Li et al., 1993; Li et al., 2000), free radical scavenging, antioxidant properties (Gao et al., 1999), and the anxiolytic effect (Wang et al., 2008). Baicalin inhibits the reverse transcriptase activity in human immunodeficiency virus-1 (HIV-1)-infected cells (Kitamura et al., 1998; Li et al., 1993), and interferes with the interaction of HIV-1 envelope proteins with chemokine receptors (Li et al., 2000). Baicalin also inhibits superantigenic staphylococcal exotoxins (SE)-stimulated T-cell proliferation, and suppresses production of inflammatory cytokines and

chemokines, such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF), interferon γ, monocyte chemotactic protein 1, macrophage inflammatory protein (MIP)-1α, and MIP-1β (Krakauer et al., 2001). In addition, baicalin dose-dependently attenuates TNF-α, IL-1β, and IL-6 expressions in paw exudates after carrageenan injection, suggesting the mechanism of inhibition of proinflammatory cytokines by baicalin (Chou et al., 2003). Signal cascades of TNF-nuclear factor-κB (NF-κB) in caerulein-induced acute pancreatitis are also suppressed by baicalin (Xue et al., 2006).

Pharmacokinetic distributions of baicalin in the striatum, thalamus and hippocampus are prominently observed after i.p. administration of *Scutellariae* Radix extract with a high content of baicalin (Zhang et al., 2006), suggesting that baicalin can pass through the blood brain barrier (BBB) and act as an active ingredient in the CNS. The effects of baicalin in the CNS have been demonstrated. Baicalin exhibits potent anti-inflammatory and anti-apoptotic effects to alleviate cerebral ischemia injury (Tu et al., 2009), in addition to the effects of cytokine suppression as aforementioned (Krakauer et al., 2001, Chou et al., 2003, Xue et al., 2006). Several lines of evidence implicate the effects of TNF-α and IL-1 in physiological sleep regulation (Krueger et al., 1999; Opp and Krueger 1992). Our previous studies have also demonstrated that the increase of proinflammatory cytokines, e.g. IL-1 and TNF-α, after microglial activation in Parkinsonism rats is dominant in the sleep disruption (Yi et al., 2007; Lu et al., 2010). Thus, the anti-inflammatory feature of baicalin may alter spontaneous sleep-wake activity. The anxiolytic-like effect of baicalin via activation of γ-aminobutyric acid (GABA)-A receptors has also been elucidated (Wang et al., 2008). GABA, an amino acid which is the major inhibitory neurotransmitter in the brain,

has long been thought to play an important role in sleep regulation (Jones 2005). It is well known that neurons containing GABA in the hypothalamus and basal forebrain, which give rise to cortical projections, mediate the induction or maintenance of slow-wave sleep (SWS) (Vincent et al., 1983). We therefore designed our current study to determine the effects of baicalin in sleep-wake activities and to elucidate the involvement of $GABA_A$ receptor activation and suppression of proinflammatory cytokines in baicalin-induced sleep regulations.

2. MATERIALS AND METHODS

2.1. Substances. Stock solutions of human recombinant IL-1β (Bachem, Torrance, CA, USA) and bicuculline methobromide (Tocris, Bristol, UK) were dissolved in pyrogen-free saline (PFS), and baicalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in 0.5 % of dimethyl sulfoxide (0.5 % DMSO, Sigma-Aldrich, St. Louis, MO). These stock solutions were stored at -20 °C until administration. The doses of the substances used in these experiments were as follows: 42.7 and 85.3 ng (50 and 100 μM, respectively) for baicalin; 2.5 ng for IL-1β; 0.1 and 0.5 ng for bicuculline. All of the administration routes were through intracerebroventricular (ICV) injection, and the total volume for each administration was 3 μl. Substances were ICV administered 20 minutes either prior to the light onset or before the dark onset.

2.2. Animals. Male Sprague-Dawley 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 an analgesic (morphine) and an antibiotic (penicillin G benzathine). Rats were surgically implanted with three electroencephalogram (EEG) screw electrodes (on the right hemisphere of the frontal and parietal lobes and the left hemisphere of the occipital lobe) and a guide cannulae directed into the lateral ventricle, as previously described (Chang and Opp, 1998). The coordinates for this procedure (AP: -1.0 mm from bregma; ML: 1.6 mm; DV: 3.5 mm) were adopted from the Paxinos and Waton rat atlas (Paxinos and Waton 1998). Insulated leads from EEG electrodes were routed to a Teflon pedestal (Plastics One, Roanoke, VA, USA). The Teflon pedestal was then cemented to the skull with dental acrylic (Tempron, GC Co., Tokyo, Japan). 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 an 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 Institutional Animal Care and Use Committee of National Taiwan University.

On the second postsurgical day, the rats were connected to the recording apparatus (see later) via a flexible tether. Three days after surgery, the patency and free drainage of the ICV cannulae was assessed by administering 200 - 400 ng of angiotensin II [human angiotensin II octapeptide; Tocris Cookson, Inc.]; angiotensin elicits a drinking response mediated by structures in the preoptic area (Epstein et al., 1970). At the end of each experimental protocol, the rats were again injected with angiotensin; only data from

the rats that exhibited a positive drinking response were included in the subsequent analyses. Animals were habituated by daily handling and ICV injections of PFS timed to coincide with scheduled experimental administrations.

2.3. 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 analog bandpass was 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, pending subsequent analyses. All recordings started at either the beginning of the light period or the beginning of the dark period and lasted for 23 hours.

Postacquisition determination of the 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 (Chang and Opp 1998). 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.

2.4. Experimental Protocol. A total of 42 Sprague-Dawley rats were used and divided into five groups, as depicted in Figure 1. Eight rats in group 1 were used to demonstrate the effect of baicalin during the light period. An undisturbed baseline recording was acquired on the first day of experiment and lasted for 23 hours. Rats then received vehicle (0.5 % DMSO), 50 μM baicalin and 100 μM baicalin over the following consecutive days, and EEG and sleep recordings were obtained for 23 hours every day. Rats in group 2 (n = 8) received the same protocol as those in group 1, except the substances were administered 20 minutes prior to the dark period, and the recordings started from the dark onset. The obtained results elucidated bacalin's effect in the dark period. Group 3 (n = 8) was designed to elicit the involvement of IL-1β in baicalin-induced sleep alteration. Vehicle administrations were given on the second and third days. IL-1β was injected 20 minutes prior to the dark onset in the following days, and two doses (50 and 100 μM) of baicalin were co-administered with IL-1β to demonstrate its antagonist effect on IL-1β-induced sleep. Rats in group 4 (n = 12) received either 0.5 % of DMSO (n = 6) or 100 μM of baicalin (n = 6) 20 minute prior to the light onset, and were decapitated 2 hours after administration. Three distinct brain regions, including the hypothalamus, hippocampus and cerebral cortex, were dissected and frozen in -80 ℃ until

IL-1β enzyme-linked immunosorbent assay (ELISA) (as described in the following). Rats in group 5 (n = 6) were employed to demonstrate the involvement of GABA in baicalin-induced sleep alteration during the dark period. The rats respectively received 0.5 % of DMSO and 100 μM of baicalin at the beginning of the dark onset on the second and third days, and PFS was administered in the middle of the dark period (at the beginning of hour-6 of the dark period) on both days. Two doses of bicuculline (0.1 and 0.5 ng) were injected in the middle of the dark period, in addition to the baicalin received at the beginning of the dark period on the fourth and fifth days.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA) for IL-1β. The rat IL-1β ELISA kits were obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA), and the procedure was carried out according to the standard instruction provided by the manufacturer. The absorbance was measured by an ELISA plate reader (Multiskan EX, Thermo Electron Corp., Waltham, MA, USA) that set the wavelength at 450 nm and 550 nm. According to the manufacturer's specifications, the sensitivity is < 12 pg/ml, and the assay range is between 25.5 ~ 2500 pg/ml; intra-assay precisions of variation are between 2.9 % and 11.6 %, and intra-assay precisions of variation are between 6.2 % and 12.3 %. This ELISA kit is specific for the measurement of natural and recombinant rat IL-1β, and does not cross-react with rat IFNγ, IL-1α, IL-2, IL-4, IL-6, IL-10, MCP-1, MIP-1α, or RANTES.

2.6. Statistical Analyses for experiment protocol. All values were presented as the mean ± SEM for the indicated sample sizes. One-way analyses of variance (ANOVA) for the duration of each vigilance state

(SWS, REMS, WAKE) and for sleep architecture parameters were performed across the two 12-h time blocks or between the specific time blocks. If statistically significant differences were detected, post hoc (*Scheffe*) comparisons were made to determine which hourly intervals, during experimental conditions, deviated from the values obtained from the same animals during controlled conditions. Results of IL-1β ELISA were compared between the values obtained after DMSO and concentrations obtained after baicalin administration by using an unpaired student t-test. An α level of $p \leq 0.05$ was taken as indicating a statistically significant difference between vehicle and active substances.

3. RESULTS

3.1. The biphasic effects of baicalin on sleep regulation.

Intracerebroventricular administration of vehicle (0.5 % DMSO) exhibited no effect on both SWS and REMS during the light period, as shown in Figure 2. Administration of baicalin 20 minutes prior to the beginning of the light period significantly suppressed SWS during the first two hours of the light period, especially during the first hour after the injection. The percentages of time spent in SWS were significantly decreased from 62.2 ± 4.2 % (obtained after the DMSO administration) to 48.7 ± 7.8 % and 47.4 ± 7.3 % after receiving 50 and 100 μM of baicalin, respectively ($n = 8$; $p < 0.05$, Figure 2). A rebound of SWS enhancement was observed during hour 4 after baicalin injection. A mirror effect of wakefulness enhancement was observed during the first hour after baicalin. REMS was not significantly altered by baicalin. In contrast to the suppression of SWS by baicalin during the light period, ICV administration of baicalin 20 minutes prior to the dark onset significantly increased SWS during hours

8-10 of the dark period, as shown in Figure 3. The percentage of time spent in SWS was increased from 19.2 ± 1.8 % (acquired after DMSO administration) to 25.4 ± 2.1 % and 33.1 ± 2.2 % after administering 50 and 100 μM of baicalin, respectively ($n = 8$; $p < 0.05$, Figure 3). Furthermore, 100 μM of baicalin also significantly increased REMS during hours 8-10 of the dark period; the percentage of time spent in REMS was enhanced from 5.6 ± 1.0 % (obtained after DMSO) to 9.8 ± 1.0 % after baicalin administration ($p< 0.05$, Figure 3). These observations indicate biphasic effects of baicalin on sleep-wake regulations; the suppression of SWS during the light (rest) period and enhancement of both SWS and REMS during the dark (active) period.

The analyses of sleep architecture parameters across hours 1 to 2 revealed that the decrease of SWS and the enhancement of wakefulness during the light period induced by baicalin was primarily due to an increase in waking bout duration (Table 1). There was a decreasing trend for both SWS bout numbers and duration, although only the decrease of SWS bout duration after administration of 50 μM baicalin reached statistical significance (Table 1). The number of transitions from one state of vigilance to another during the 2-hour light period was not altered, which indicates sleep was not fragmented (Table 1). In contrast, the enhancement of SWS during hours 8~10 of the dark period, after administrating baicalin at the beginning of the dark period, was primarily due to an increase of SWS bout numbers (Table 1). Baicalin-induced REMS enhancement was caused by the increase of both REMS bout numbers and duration (Table 1). In addition, the number of transitions from one state of vigilance to another during hours 8~10 of the dark period was increased, indicating the fragmentation of sleep (Table 1).

3.2. Baicalin suppresses IL-1β-induced SWS during the dark period.

We first would like to elucidate whether the effect of SWS suppression induced by baicalin during the light period is primarily due to the blockade of IL-1. The somnogenic properties of IL-1 and its circadian fluctuation are well documented [reviewed in (Krueger and Fang 1999) and (Opp and Krueger 1992)]. IL-1 administered centrally into rats during their active period (dark period) is particularly effective in increasing SWS and reducing waking. IL-1β mRNA expressions in rat brains are highest during the light period of the light-dark cycle (the period when rats sleep the most), and lowest during the dark period, when rats are most active. Therefore, we designed the following experiments to investigate the pharmacologically blockade effect of baicalin on exogenous IL-1-induced sleep during the dark period. We confirmed that the administration of IL-1β 2.5 ng before the beginning of the dark period increased SWS during hours 1~6; IL-1β significantly increased SWS from 15.6 ± 2.4 %, obtained after vehicle (DMSO + PFS), to 28.5 ± 3.5 % ($n = 8$; $p < 0.05$, Figure 4). However, IL-1β did not alter REMS during the dark period. Our results further demonstrated that baicalin dose-dependently blocked IL-1β-induced SWS enhancement. The percentage of time spent in SWS after receiving 100 μM baicalin was significantly reduced to 19.6 ± 2.3 % ($p < 0.05$), when compared to that obtained after IL-1β (Figure 4). This observation suggests that baicalin possesses ability to antagonize IL-1β action. Therefore, baicalin-induced SWS decrease during the light period may be due to its antagonism to the IL-1 effect, since IL-1β exhibits its highest concentration of circadian fluctuation during the light period of the light:dark cycle (Opp and Krueger 1992). During hours 9-10 of the dark period, the amount of SWS – but

not REMS – exhibited a tendency of increase from 13.7 ± 4.1 %, obtained after vehicle (DMSO + PFS), to 20.0 ± 4.2 % when baicalin was injected in addition to the IL-1β administration; however the change did not reach statistical significance.

We have further clarified whether IL-1 concentrations in brain are altered by baicalin. Our results, as shown in Figure 5, indicated that IL-1β expressions in several brain regions, including the hypothalamus, hippocampus and cortex, were not significantly altered during the light period after 100 μM baicalin administration, suggesting baicalin has no effect on the regulation of IL-1β expressions.

3.3. Bicuculline blocked baicalin-induced SWS and REMS enhancement during the dark period.

We then further determined whether the enhancement of SWS and REMS by baicalin during the dark period involves the GABAergic system. Baicalin-induced enhancements in SWS and REMS during the dark period were also observed in this group of rats; both SWS and REMS were significantly increased during hours 8~9 (Figure 6). The amount of SWS during hours 8~9 was increased from 20.0 ± 4.2 % (obtained after DMSO) to 31.2 ± 3.0 % after baicalin administration ($n = 6$, $p < 0.05$). REMS was also enhanced from 3.5 ± 1.3 % after DMSO to 7.9 ± 1.7 % after administration of baicalin ($p < 0.05$). The administration of $GABA_A$ receptor antagonist bicuculline (0.5 ng) in the middle of the dark period significantly blocked baicalin-induced enhancement of SWS and REMS. The percentages of time spent in SWS and REMS during hours 8~9 were reduced to 12.6 ± 3.8 % and 2.6 ± 1.1 %, respectively, after administering bicuculline in the middle of the dark period ($p < 0.05$, when compared to the values

obtained after baicalin administration, Figure 6).

4. DISCUSSION

Scutellariae Radix (Huang Qin), a traditional Chinese medicine, has been widely used as a major component in prescriptions for anti-febrile and detoxification medications, according to ancient Chinese literature. Additional pharmacological effects including anti-inflammation, anti-bacteria, anti-hypertension and anti-allergy have been described, as aforementioned. However, the CNS functions of *Scutellariae* Radix have been less emphasized, especially in relation to its sedative and hypnotic effects. Flavonoids are found in almost all of the plants, and exhibit their pharmacological activities as they are the major constituents in many plant medicines. Four major flavonoids with the basic backbone of 2-phenyl-1,4-benzopyrone have been purified from *Scutellariae* Radix, including baicalin, baicalein, wogonoside, and wogonin. Since they possess the same structural backbone, these four flavonoids have similar pharmacological effects. For example, both wogonin and baicalin have been reported to interact with the benzodiazepine site of $GABA_A$ receptors to exhibit the anxiolytic effect (Hui et al., 2002; Wang et al., 2008); anti-inflammatory effects of baicalin, baicalein, and wogonin have been well documented (Kubo et al., 1984; Sekiya and Okuda, 1982; Wakabayashi 1999); wogonin diminishes lipopolysaccharide (LPS)-induced TNF-α and IL-1β production in a dose-dependent manner in the BV-2 mouse microglia cells or in rat primary microglial cultures (Lee et al., 2003); and baicalin dose-dependently attenuates TNF-α and IL-1β formation in paw exudates after carrageenan-induced inflammation (Chou et al., 2003). Baicalin, as a pharmacological active compound, has been proved to pharmacokinetically distribute,

without via metabolism, into several brain regions, including the cerebral cortex, hippocampus, striatum, thalamus and brain stem, after intravenous administration of baicalin-enriched *Scutellariae* Radix extract into rats (Zhang etal., 2006). As a result, this study was designed to investigate the CNS role of baicalin on the sleep-wake regulation. We determined the effects of the sleep-wake regulation induced by baicalin during the dark period and during the light period. The rodent is a nocturnal animal which sleep more during the light (rest) period and is more active in the dark (active) period. Our results demonstrated the biphasic and paradoxical effects of sleep alteration; administration of baicalin at the beginning of the light period significantly reduced SWS and enhanced wakefulness, without altering REMS, during the first two hours after injection, whereas both SWS and REMS were increased and wakefulness was reduced during the dark period, when rats received baicalin before the dark onset. This observation explicated the cruciality of circadian time points for giving baicalin in determining the direction of sleep changes. Our unpublished observation has also revealed that wogonin exhibits the similar biphasic sleep effects, with a decrease of SWS during the light period and an increase of SWS during the dark period (unpublished data). This result may also explain why the dominant effects of *Scutellaria baicalensis* Georgi described in ancient Chinese literature did not include the hypnotic effect, due to its contradictory effects on sleep.

The somnogenic effects of IL-1β and TNF-α have been well documented in literature (Krueger and Fang 1999; Opp and Krueger 1992). Messenger RNA expression of IL-1β and TNF-α in the rat brain is highest during the light period of the light-dark cycle, the period when rats sleep the most, and lowest during the dark period, when rats are most active. IL-1β administered centrally into rats during their active period

(dark period) is particularly effective in increasing SWS and reducing waking (Krueger and Fang 1999; Opp and Krueger 1992). Besides their effects on physiological sleep regulation, proinflammatory cytokines, including IL-1β and TNF-α, have previously depicted the implication of pathological sleep disruption caused by neurological diseases, such as epilepsy and Parkinson's disease (Yi et al, 2004; Yi et al., 2007; Lu et al., 2010). Because the expression of IL-1β is highest during the light period and baicalin possesses the ability to suppress IL-1's effect (as aforementioned), we speculated that the baicalin-induced SWS decrement during the first two hours of the light period is due to the blockade of IL-1 receptors, the decrease of IL-1 production, or both. Therefore, we first determined its ability to block exogenous IL-1β-induced SWS enhancement during the dark period when the expression of IL-1β and the amount of SWS are lowest. Our results have shown that SWS was increased during the first 6 hours of the dark period after IL-1β administration, and this IL-1β-induced SWS enhancement was dose-dependently blocked by baicalin. This suggests baicalin can pharmacologically block IL-1-mediated SWS alteration, providing evidence that the decrease of SWS during the light period by baicalin may be due to the pharmacological blockade of IL-1β. Because in this experiment baicalin was administered at the beginning of the dark period, we also discovered SWS enhancement during hours 9~10 of the dark period. The percentage of time spent in SWS was increased from 13.7 ± 4.1 % (obtained after vehicles) to 20.0 ± 4.2 % after co-administration of baicalin and IL-1β (Figure 4), which is similar to baicalin's results observed in the dark period group in Figure 3, though the change of SWS did not reach statistical significance and REMS was not altered. Baicalin displays its pharmacological effects on the blockade of IL-1β-mediated SWS enhancement during the early 6 hours of the dark period, so that with the same

amount of dosage, the enhancement of SWS by baicalin during the dark period will be lessened. The reason why REMS enhancement during the dark period after administration of baicalin was not observed could be due to the effect of IL-1β on REMS suppression (Opp and Krueger 1992). Because the REMS is at a minimal level during the dark period, the IL-1β-mediated REMS suppression is not obviously, but the baicalin-induced REMS increase might be suppressed by IL-1β. Nevertheless, this speculation needs to be further confirmed in a future study. Since evidence indicates that wogonin diminishes lipopolysaccharide (LPS)-induced TNF-α and IL-1β (Lee et al., 2003) and baicalin attenuates TNF-α and IL-1β formation after inflammation (Chou et al., 2003), we herein further determined whether the suppression of IL-1β expression during the light period is involved in the baicalin-induced SWS enhancement. However, we demonstrated that the IL-1β concentrations in the hypothalamus, hippocampus, and cerebral cortex were not significantly altered by ICV administration of baicalin, as shown in Figure 5. Collectively, the effect of baicalin on SWS enhancement during the light period is primarily due to the pharmacological blockade of IL-1 rather than the suppression of IL-1 production.

Baicalin has been demonstrated to create an anxiolytic-like effect via the pharmacological actions of $GABA_A$ receptors in a Vogel conflict test and elevated plus maze test (Liao et al., 2003; Xu et al., 2006). Baicalin has been found to interact with benzodiazepine binding site of the $GABA_A$ receptors with a *Ki* value of 77.1 μM, although its affinity for benzodiazepine binding site is less than that of wogonin and baicalein (Hui et al., 2000). Wang et al. (2008) further clarified that baicalin prefers to bind to α2- and α3-containing $GABA_A$ receptor subtypes, compared to α1- and α5-containing subtypes, suggesting α2-

and α3-containing $GABA_A$ receptor subtypes are important drug targets for the baicalin-induced anxiolytic effect (Wang et al., 2008). We have demonstrated that ICV administration of baicalin at the beginning of the dark period enhanced both SWS and REMS during hours 8~10, as shown in Figures 3 and 6, and the injection of $GABA_A$ receptor antagonist bicuculline in the middle of the dark period significantly blocked the baicalin-induced enhancements of SWS and REMS, suggesting that this effect is mediated by $GABA_A$ receptors. This observation further confirms that the constituent of *Scutellariae* Radix, baicalin, possesses somnogenic effects through interacting with the benzodiazepine site of $GABA_A$ receptors, although we still don't understand why it needs to take 8 hours to exhibit its somnogenic function. The reason may be due to its relative low affinity for benzodiazepine binding sites; it is of worth to conduct an investigation on this issue.

CONCLUSION

Our findings suggested that baicalin exhibits biphasic effects on the sleep-wake regulation; the decrease of SWS during light period and an increase of SWS and REMS during dark period. Inhibition of IL-1β action and the enhancement of $GABA_A$ receptor activity may respectively mediate baicalin's effects during the light and dark period.

FIGURE LEGENDS

Figure 1. The diagram for the experimental protocol. Close bar indicates the dark period and the open bar represents the light period of the 12:12h light:dark cycle. Arrow depicts either the timing of ICV injection or the sacrifice time for animals. The symbol "R" depicts the recording.

Figure 2. The effect of baicalin on sleep-wake activity during the light period. Shade depicts the values obtained after DMSO vehicle control, open circles indicate the values obtained after 50 μM baicalin administration, and closed circles represent the data obtained after 100 μM baicalin injection. The bar histogram elucidates the averaged values of time spent in SWS obtained during the time blocks of hours 1~2. Those bars from the left to the right depict the values acquired after baseline (undisturbed rats), DMSO administration, 50 μM of baicalin, and 100 μM of baicalin, respectively. The values were presented as mean ± SEM. * represents a statistically significant difference between the values obtained from the DMSO vehicle control and baicalin treatment. The dark and open portions of horizontal bars represent the dark and light periods of the 12:12h light: dark cycle. WAKE refers to wakefulness; REMS, rapid eye movement sleep; SWS, slow-wave sleep. LD: low dose (50 μM of baicalin); HD: high dose: (100 μM of baicalin).

Figure 3. The effect of baicalin on sleep-wake activity during the dark period. Shade depicts the values obtained after DMSO vehicle control, open circles indicate the values obtained after 50 μM baicalin

administration, and closed circles represent the data obtained after 100 μM baicalin injection. The bar histograms elucidate the averaged percentages of time spent in SWS (the upper panel) and in REMS (the lower panel) obtained during the time blocks of hours 8~10. Those bars from the left to the right depict the values acquired after baseline (undisturbed rats), DMSO administration, 50 μM of baicalin, and 100 μM of baicalin, respectively. The values were presented as mean ± SEM. * represents a statistically significant difference between the values obtained from the DMSO vehicle control and baicalin treatment. The dark portion of horizontal bar represents the dark period of the 12:12h light:dark cycle. WAKE refers to wakefulness; REMS, rapid eye movement sleep; SWS, slow-wave sleep. LD: low dose (50 μM of baicalin); HD: high dose: (100 μM of baicalin).

Figure 4. Baicalin blocks IL-1β-induced SWS enhancement during the dark period. Shade depicts the values obtained after vehicle control (DMSO+PFS), closed circles indicate the values obtained after IL-1β administration, open triangles represent the values after co-administering 50 μM baicalin+IL-1β, and closed triangles demonstrate the data obtained after administration of 100 μM baicalin+IL-1β. The bar histograms elucidate the averaged percentages of time spent in SWS obtained during the time blocks of hours 1~6. Those bars from the left to the right depict the values acquired after vehicles (PFS+PFS and DMSO+PFS), DMSO+IL-1β, 50 μM baicalin+IL-1β, and 100 μM baicalin+IL-1β, respectively. The values were presented as mean ± SEM. # represents a statistically significant difference between the values obtained from vehicle control (DMSO+PFS) and DMSO+IL-1β treatment. * represents a statistically significant difference between the values obtained after DMSO+IL-1β and 100 μM

baicalin+IL-1β. The dark portion of horizontal bar represents the dark period of the 12:12h light:dark cycle. WAKE refers to wakefulness; REMS, rapid eye movement sleep; SWS, slow-wave sleep. LD: low dose (50 μM of baicalin); HD: high dose: (100 μM of baicalin).

Figure 5. Baicalin does not alter IL-1β concentrations in the hypothalamus, hippocampus, and cortex. Open bars represent the values obtained after DMSO treatment, and the hatched bars demonstrate the concentrations obtained after receiving 100 μM of baicalin.

Figure 6. $GABA_A$ receptor antagonist, bicuculline, blocks baicalin-induced SWS and REMS enhancements during the dark period. DMSO and baicalin were administered 20 minutes prior to the beginning of the dark period, and PFS and bicuculline were injected in the middle of the dark period (at hour-6; the arrows indicate the injection time points). Open circles depict the values obtained after DMSO+PFS control, closed circles indicate the values obtained after 100 μM baicalin+PFS administration, and closed triangles represent the data obtained after 100 μM baicalin+0.5 ng bicuculline. The bar histograms elucidate the averaged percentages of time spent in SWS (the upper panel) and in REMS (the lower panel) obtained during the time blocks of hours 8~9. Those bars from the left to the right depict the values acquired after receiving vehicles (DMSO+PFS), 100 μM baicalin+PFS, 100 μM baicalin+0.1 ng bicuculline, and 100 μM baicalin+0.5 ng bicuculline, respectively. The values were presented as mean ± SEM. * represents a statistically significant difference between the values obtained from vehicles (DMSO+PFS) and 100 μM baicalin+PFS treatment. # depicts a statistically significant

difference between the values obtained after receiving 100 μM baicalin+PFS and 100 μM baicalin+0.5 ng bicuculline treatment. WAKE refers to wakefulness; REMS, rapid eye movement sleep; SWS, slow-wave sleep. LD: low dose (0.1 ng of bicuculline); HD: high dose: (0.5 ng of bicuculline).

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Table

Table 1. Effects of baicalin on sleep-wake architecture parameters of rats.

Manipulation[4]	Hour	L:D Cycle[5]	Number of bouts[1] WAKE[6]	Number of bouts[1] SWS[6]	Number of bouts[1] REMS[6]	Bout duration[2] WAKE	Bout duration[2] SWS	Bout duration[2] REMS	Transitions[3]
Vehicle control (PFS)	1-2	L	3.9 ± 0.5	7.3 ± 0.9	2.8 ± 0.5	6.9 ± 3.5	5.2 ± 0.7	1.4 ± 0.1	38.5 ± 5.2
Vehicle control (DMSO)	1-2	L	5.2 ± 0.7	8.0 ± 1.1	3.1 ± 0.5	4.4 ± 1.6	5.1 ± 0.6	1.3 ± 0.2	42.1 ± 5.0
Baicalin 50 μM	1-2	L	3.7 ± 0.4	7.2 ± 0.8	3.0 ± 0.7	11.7 ± 4.5	3.6 ± 0.6*	1.1 ± 0.2	30.4 ± 4.4
Baicalin 100 μM	1-2	L	4.5 ± 0.9	5.9 ± 1.2	1.5 ± 0.4*	14.4 ± 6.2*	4.5 ± 0.6	1.1 ± 0.2	33.1 ± 7.2
Vehicle control (PFS)	8-10	D	3.8 ± 0.3	5.3 ± 0.4	3.0 ± 0.4	15.3 ± 2.3	1.7 ± 0.1	0.8 ± 0.1	32.5 ± 2.7
Vehicle control (DMSO)	8-10	D	4.0 ± 0.4	5.8 ± 0.6	2.6 ± 0.4	17.2 ± 3.6	1.7 ± 0.2	0.8 ± 0.1	31.9 ± 3.4
Baicalin 50 μM	8-10	D	4.8 ± 0.5	6.8 ± 0.5	3.7 ± 0.5	13.5 ± 2.3	2.0 ± 0.2	1.1 ± 0.1	36.8 ± 3.8
Baicalin 100 μM	8-10	D	6.8 ± 0.7*	9.4 ± 0.9*	4.3 ± 0.4*	6.2 ± 0.9*	2.1 ± 0.2	1.3 ± 0.1*	45.0 ± 2.8*

Values are Means ± S.E.M. * denotes a statistically significant difference ($p < 0.05$) between values obtained after administration of vehicle (DMSO) and those obtained after receiving baicalin treatment.

[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
[5] Period of the light:dark cycle immediately prior to which injections were given: D = dark period.
[6] Vigilance states: WAKE, wakefulness; SWS, slow-wave sleep; REMS, rapid eye movements sleep

Figure

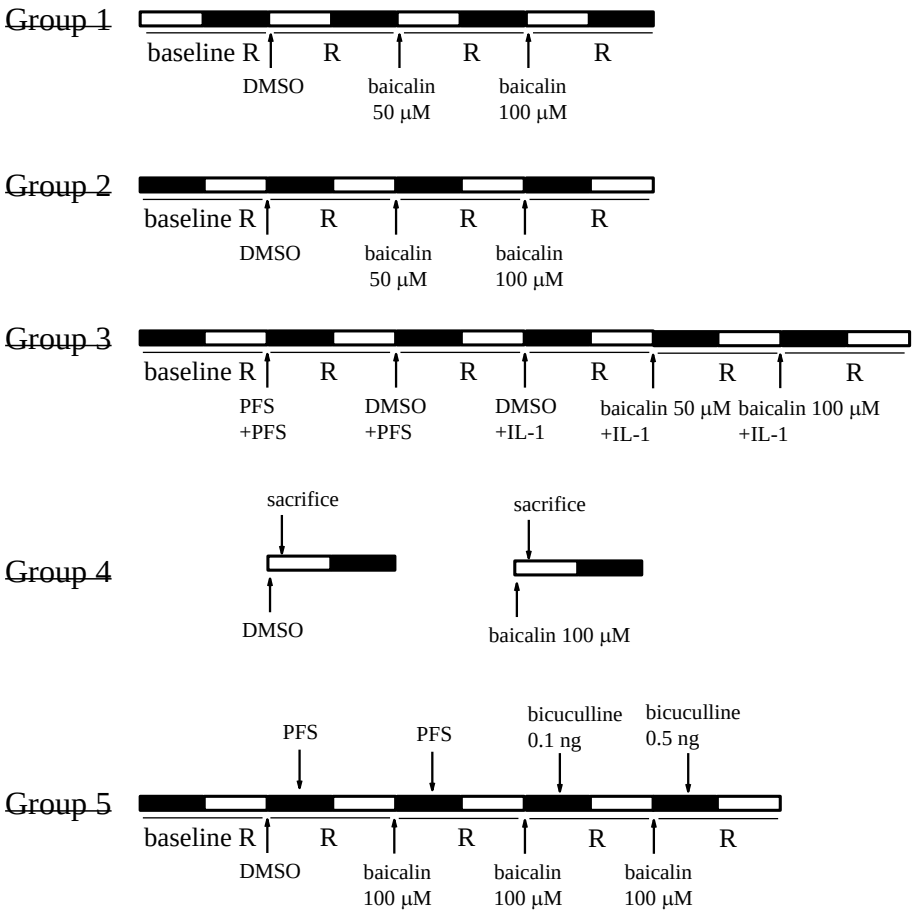

Figure 1

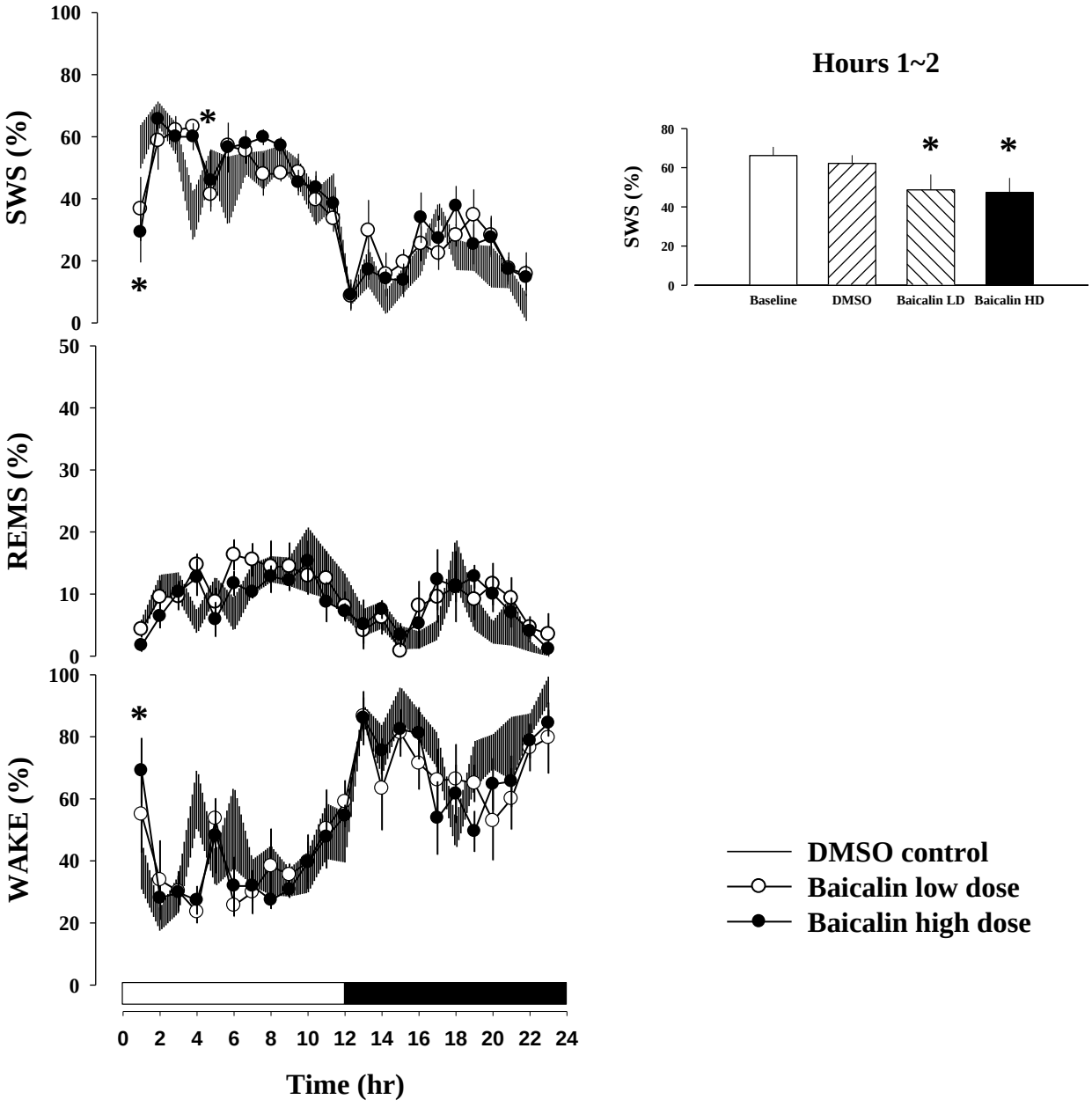
SWS (%)
REMS (%)
WAKE (%)
Time (hr)
Hours 1~2
Baseline
DMSO
Baicalin LD
Baicalin HD
DMSO control
Baicalin low dose
Baicalin high dose

Figure 2

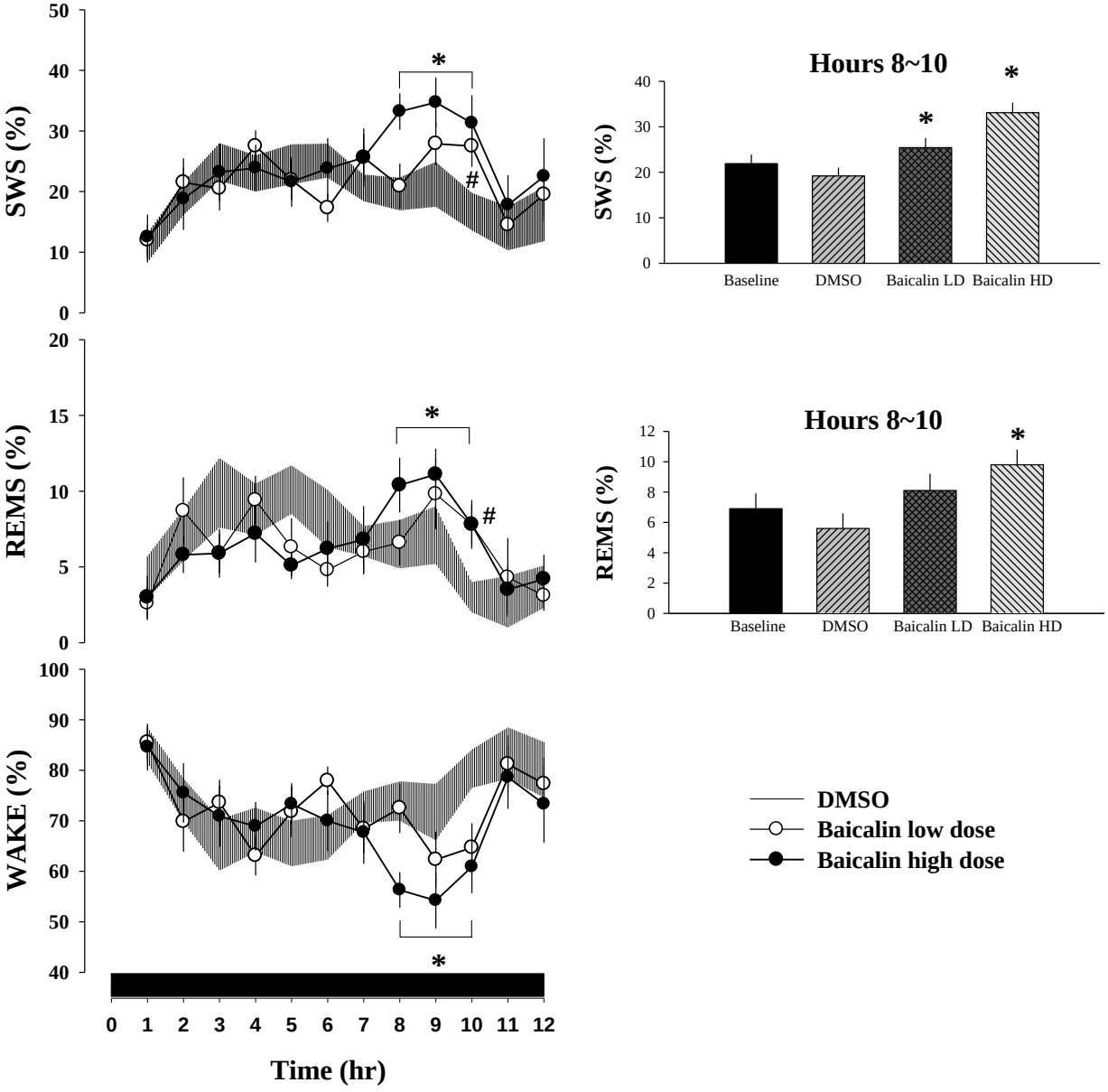
SWS (%)
REMS (%)
WAKE (%)
Time (hr)
Hours 8~10
Baseline
DMSO
Baicalin LD
Baicalin HD
DMSO
Baicalin low dose
Baicalin high dose

Figure 3

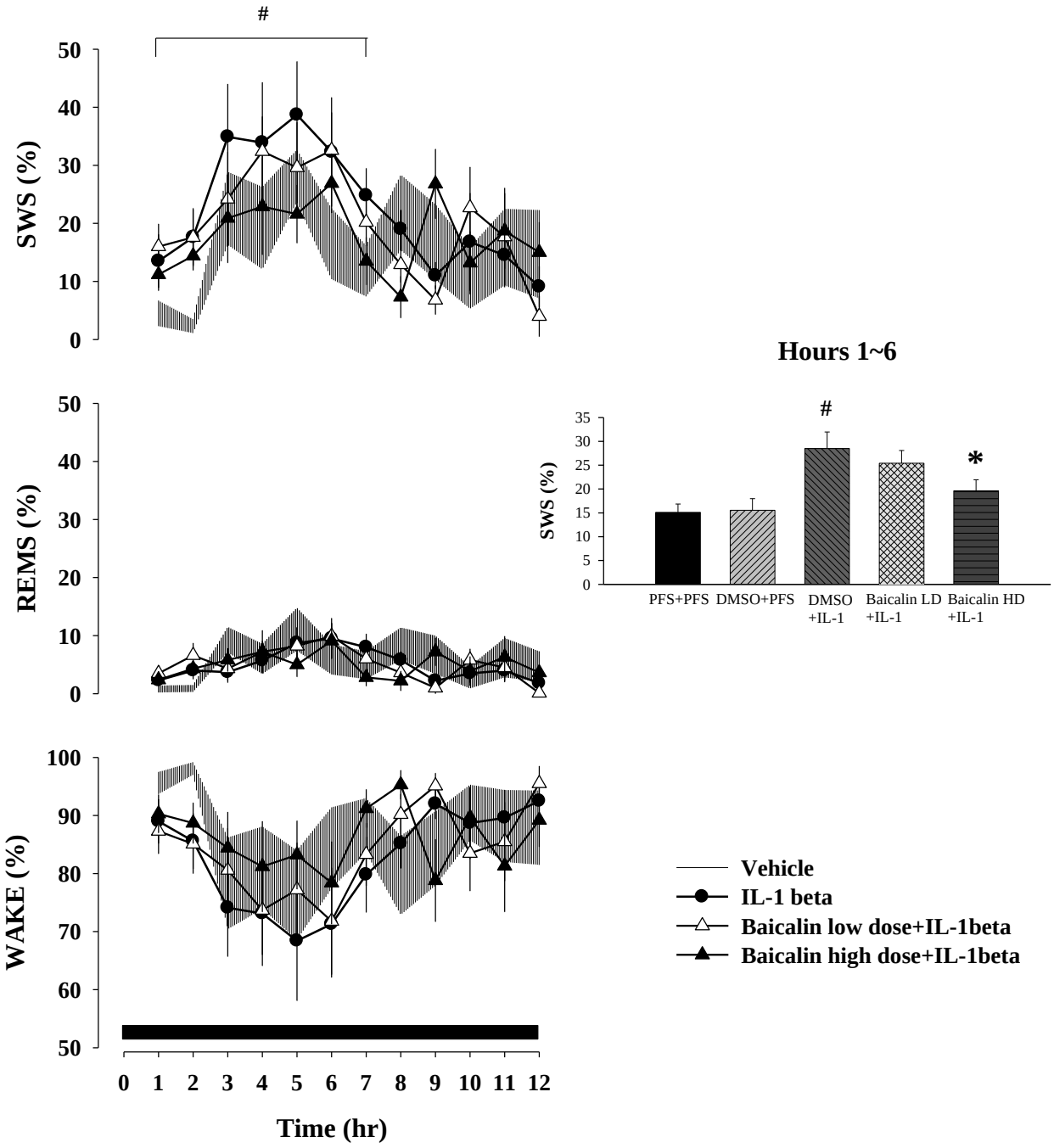
#
SWS (%)
REMS (%)
WAKE (%)
Time (hr)
Hours 1~6
SWS (%)
PFS+PFS
DMSO+PFS
DMSO +IL-1
Baicalin LD +IL-1
Baicalin HD +IL-1
Vehicle
IL-1 beta
Baicalin low dose+IL-1beta
Baicalin high dose+IL-1beta

Figure 4

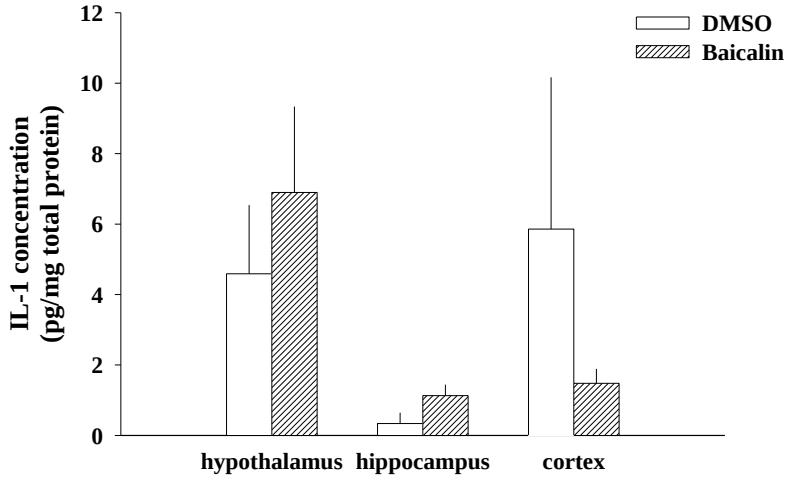
12
10
8
6
4
2
0
IL-1 concentration
(pg/mg total protein)
DMSO
Baicalin
hypothalamus
hippocampus
cortex

Figure 5

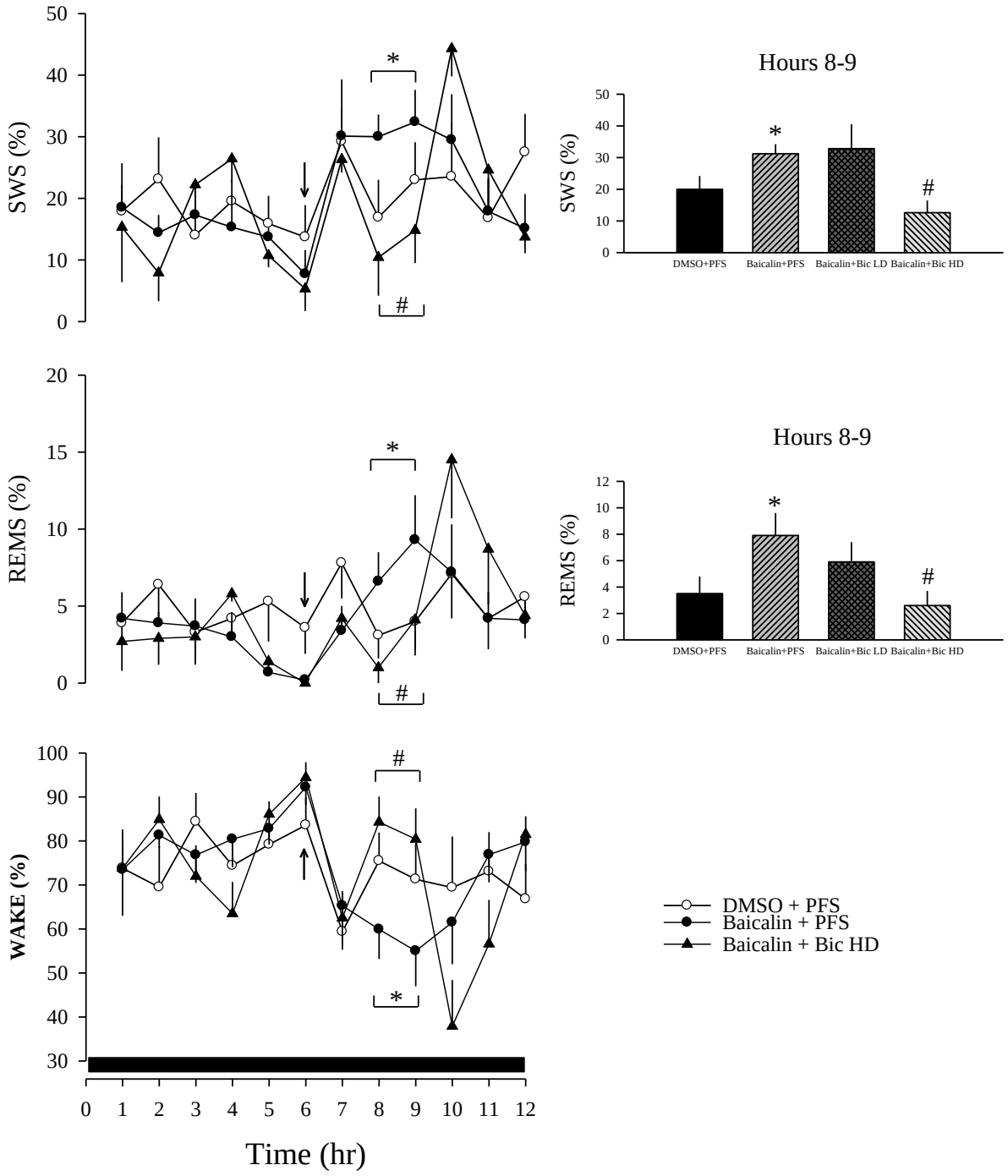
SWS (%)
REMS (%)
WAKE (%)
Time (hr)
Hours 8-9
DMSO+PFS
Baicalin+PFS
Baicalin+Bic LD
Baicalin+Bic HD
DMSO + PFS
Baicalin + PFS
Baicalin + Bic HD

Figure 6