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Caudal Nucleus Tractus Solitarius (NTS)在針灸改變睡眠所扮演的角色

The Role of Caudal Nucleus Tractus Solitarius (NTS) In Electroacupuncture-induced Sleep Activities

鄭穹翔

Chiung-Hsiang Cheng

指導教授: 張芳嘉 博士

Advisor: Fang-Chia Chang, Ph.D.

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List of Abbreviations

NTS: nucleus tractus solitaries

NREM sleep: non-rapid eye movement sleep

REM sleep: rapid eye movement sleep

PBN: parabrachial nuclei

VM: ventromedial nucleus of the thalamus

EA: electroacupuncture

SWA: slow wave activity

SWS: slow-wave sleep

EEG: electroencephalogram

CRH: corticotrophin-releasing hormone

CNS: central nervous system

IR: immunoreactivity

PFS: pyrogen-free saline

ELISA: enzyme-linked immunosorbent assay

IP: intraperitoneally

ANOVA: analysis of variance

NMDA: N-methyl-D-aspartate

LHA: lateral hypothalamic area

TEM: transmission electron microscopy

PBS: phosphate buffer saline

PVN: paraventricular nucleus

WHO: World Health Organization

Abstract in Chinese

電針刺激可以有許多治療功用，例如止痛、消炎、治療睡眠障礙。電針刺激改善睡眠的機制目前還不明瞭。在中醫理論裡：安眠穴，是一個可以改善失眠的穴道。之前發現 10Hz 電針刺激安眠穴可以使孤獨徑核 (nucleus tractus solitaries, NTS) 的乙醯膽鹼性神經活化，造成大鼠在暗期的非快速動眼睡眠(non-rapid eye movement sleep; NREM sleep) 上升。我們假設除了乙醯膽鹼類神經外，內生性嗎啡也可能是影響電針在睡眠的作用。本研究結果顯示，10Hz 電刺激安眠穴增加了暗期的非快速動眼睡眠但是不影響快速動眼睡眠 (rapid eye movement sleep; REM sleep)，且這些增加的 NREM 可以被注射到 NTS 的 naloxone (opioid receptor antagonist) 和 naloxonazine (μ-opioid receptor antagonist 拮抗) 抑制。而注射 natrindole (σ-opioid receptor) 和 nor-binaltrophimine (κ-opioid receptor) 則沒有作用。除此之外 β-endorphin 的表現量在 10Hz 電針刺激後於腦幹和海馬迴都有上升且會被注射到 NTS 的 muscarinic antagonist: scopolamine 拮抗。我們的結果顯示 10Hz 電刺激安眠穴增加的非快速動眼可能是藉由乙醯膽鹼活化後使 opiodergic 神經分泌 β-endorphin 增高，作用在 μ-opioid receptor。

解剖上，從 NTS 來的神經投射到 parabrachial nuclei (PBN)，再投射到視丘的腹內側神經核 (ventromedial nucleus of the thalamus; VM)；或直接由 NTS 投射到視丘的腹內側神經核。清醒時腦中皮質的神經突觸活性較強，但在睡眠時的神經活動基本上會減少這些突觸的活性。從 NTS 到 PBN 以及 VM 的嗎啡類神經藉由嗎啡類受體過極化了 PBN 以及 VM 中的神經。因此我們假設 10 Hz 電針刺激增加 NTS 的突觸活性，造成 PBN 以及 VM 的過極化，導致增加睡眠的現象。我們接著研究 10 Hz 電針刺激安眠穴後，NTS 和 VM 的突觸密度和長度的改變。我們發現 10 Hz 電針刺激安眠穴後 NTS 和 VM 的突觸密度都有增加，但是突觸總長度只有在 NTS 部位有顯著改變。這實驗結果可能代表了電針刺激會增加興奮性突觸的長度以及密度來增強 NTS 的突觸強度，並且在 VM 增加抑制性突觸的

密度來減低 VM 的突觸強度，並藉此達到電針安眠穴增加睡眠的作用。

有報告指出不同頻率的電針刺激可以增加不同種的內源性嗎啡，且作用在不同的類嗎啡受體上。因此我們接著使用 100 Hz 的電針刺激安眠穴發現也增加非快速動眼睡眠，但不會影響快速動眼睡眠。100Hz 電刺激增加的非快速動眼睡眠會因為注射 naloxone (opioid receptor antagonist)和 nor-binaltrophimine (κ-opioid receptor)到 NTS 而抑制，但注射 naloxonazine (μ-opioid receptor antagonist 拮抗) 和 natrindole (σ-opioid receptor) 不會影響非快速動眼睡眠。我們的結果顯示高頻率 100 Hz 的電針刺激可能會藉由 NTS 的 κ-opioid receptor 增加非快速動眼睡眠。這些結果顯示低頻率 (10Hz) 以及高頻率 (100Hz) 電刺激安眠穴均會增加非快速動眼睡眠，其機轉和電針止痛在脊髓的機制類似，低頻刺激增加 β-endorphin 在 NTS 釋放，作用在 μ-receptor；而高頻刺激則是藉由 κ-receptor。

Keywords: 電針刺激(electroacupuncture, EA), 孤獨徑核(nucleus tractus solitaries, NTS), β-endorphin, μ-opioid receptor, κ-opioid receptor, 睡眠, 突觸形態, 視丘的腹內側神經核 (ventromedial nucleus of the thalamus; VM)

Abstract

Electroacupuncture (EA) possesses various therapeutic effects, including alleviation of pain, reduction of inflammation and improvement of sleep disturbance. The mechanisms of EA on sleep improvement, however, remain to be determined. It has been stated in ancient Chinese literature that the Anmian (EX17) acupoint is one of the trigger points that alleviates insomnia. We previously demonstrated that EA stimulation of Anmian acupoints in rats during the dark period enhanced non-rapid eye movement (NREM) sleep, which involves the induction of cholinergic activity in the nucleus tractus solitarius (NTS). In addition to cholinergic activation of the NTS, activation of the endogenous opioidergic system may also be a mechanism by which acupuncture affects sleep. Therefore, this study was designed to investigate the involvement of the NTS opioidergic system in EA-induced alterations in sleep. Our present results indicate that EA of Anmian acupoints increased NREM sleep, but not rapid eye movement (REM) sleep, during the dark period in rats. This enhancement in NREM sleep was dose-dependently blocked by microinjection of opioid receptor antagonist, naloxone, and the μ-opioid receptor antagonist, naloxonazine, into the NTS; administrations of δ-receptor antagonist, natrindole, and the κ-receptor antagonist, *nor*-binaltrophimine, however, did not affect EA-induced alterations in sleep. Furthermore, β-endorphin was significantly increased in both the brainstem and hippocampus after the EA stimuli, an effect blocked by

administration of the muscarinic antagonist scopolamine into the NTS. Our findings suggest that mechanisms of EA-induced NREM sleep enhancement may be mediated, in part, by cholinergic activation, stimulation of the opiodergic neurons to increase the concentrations of β-endorphin and the involvement of the μ-opioid receptors.

One ascending projection is from NTS to the ventromedial nucleus (VM) of the thalamus (the NTS-VM pathway). Wakefulness is accompanied by synaptic potentiation in the cortical circuits, whereas slow wave activity (SWA) during slow wave sleep (SWS) promotes a generalized depression or downscaling of synaptic strength. The VM receives opioidergic inputs from NTS and the activation of opioid receptors hyperpolarize neurons of VM. Accordingly, 10 Hz EA may increase synaptic activity of NTS and subsequently hyperpolarize and downscale the synaptic strength in the VM of thalamus by inhibitory afferents, which lead to the enhancement of SWS. Enhancement of excitatory synapses in NTS and inhibitory synapses in VM may respectively contribute to the up-regulation of synaptic strength in NTS and downscaling of synaptic strength in the VM after 10 Hz EA. Our results demonstrated that the synaptic density was increased in both NTS and VM after rats received 10 Hz EA stimuli, while the enhanced synaptic length was only observed in the NTS, suggesting that 10 Hz EA altered excitatory synaptic strength of NTS and inhibitory synaptic strength of VM by changing the synaptic morphology.

Studies have shown that different kinds of endogenous opiate peptides and receptors may mediate the consequences of EA with different frequencies. Herein we further elucidated that high frequency (100 Hz) EA of Anmian enhanced NREM sleep during the dark period, but exhibited no direct effect on REM sleep. High frequency EA-induced NREM sleep enhancement was dose-dependently blocked by microinjection of naloxone or κ-receptor antagonist (*nor*-binaltrophimine) into the caudal NTS, but was affected neither by μ-(naloxonazine) nor δ-receptor antagonists (natrindole), suggesting the role of NTS κ-receptors in the enhancement of high frequency EA-induced NREM sleep. Current and previous results have combined to depict the opioid mechanisms of EA-induced sleep.

Keywords: electroacupuncture (EA), nucleus tractus solitaries (NTS), β-endorphin, μ-opioid receptor, κ-opioid receptor, sleep, synaptic morphology, ventromedial nucleus of the thalamus (VM)

Contents

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Chapter 1. General Introduction

Electroacupuncture (EA) possesses various therapeutic effects, including alleviation of pain, reduction of inflammation and improvement of sleep disturbance. The mechanisms of EA on sleep improvement, however, remain to be determined. It has been stated in ancient Chinese literature that the Anmian (EX17) acupoint is one of the trigger points that alleviates insomnia. Yi et. al previously demonstrated that EA stimulation of Anmian acupoints in rats during the dark period enhances non-rapid eye movement (non-REM;NREM) sleep, which involves the induction of cholinergic activity in the nucleus tractus solitarius (NTS). In addition to cholinergic activation of the NTS, activation of the endogenous opioidergic system may also be a mechanism by which acupuncture affects sleep. Chang and Pomeranz had revealed that relatively low doses of naloxone only block the analgesic effect induced by low-frequency (4 Hz) of electroacupuncture (EA) stimulation, but not the consequence induced by high-frequency (200 Hz) of EA (1), suggesting that different frequency of EA increases the release of various endogenous opioids. Therefore, we designed serial studies to investigate the involvement of the NTS opioidergic system in EA-induced alterations in sleep.

Electroacupuncture (EA), which consists of passing a continuous electric current through needles inserted into the acupoints to obtain the therapeutic effects, i.e.

alleviation of pain, reduction of inflammation and management of insomnia, is modified from the traditional Chinese acupuncture. Insomnia is one of the most common sleep disorders and it has been demonstrated that the effectiveness rate of acupuncture for relieving insomnia is about 90% (2,3). Several specific acupoints have been identified for insomnia treatment based upon the differentiation and signs of symptoms according to traditional Chinese medicine. Among the acupoints used, Shenmen (HT7), Sanyinjiao (SP6) and Anmian (EX17) are the most common, although other acupoints may also be used, such as Neiguan (PC6), Zusanli (ST36), Taichong (LR3), Baihui (DU20), Dazhui (DU40), Tainzhu (BL10), Bishu (BL20) and Zhongwan (RN12) (4,5).

The mechanisms by which EA functions to alleviate clinical symptoms remain largely unknown, although applications of EA have been widely described in the Chinese literature. The spinal gate-control theory (6) and the activation of central endorphin and/or monoaminergic systems (i.e. serotonin and norepinephrine) (1) have been hypothesized in mediating the EA-induced analgesia. In addition, acupuncture may reduce the inflammation-induced elevation of body temperature by suppressing hypothalamic production of proinflammatory cytokines (7). The central opioidergic and serotonergic systems also mediate the suppressive effects of acupuncture on capsaicin-induced neurogenic inflammation (8). Recent findings suggest that the induction of vagus nerve activity appears to be another significant factor for mediating

the action of acupuncture (9,10). The caudal nucleus tractus solitarius (NTS) may be activated by acupuncture, since NTS is located in the dorsomedial medulla oblongata and receives afferents primarily from the vagus and glossopharyngeal nerves (11). Ascending projections from the NTS are traced through the lateral and dorsal tegmentum and periventricular gray up to the rostral pons and midbrain, and terminate in the parabrachial nucleus, which in turn projects to the thalamus, hypothalamus, preoptic area, bed nucleus of the stria terminalis, amygdala and the frontal cortex, regions commonly belonging to the visceral-limbic forebrain (12,13). From these anatomical data, it does not appear that the predominant effect of the NTS does not appear via the reticular activating system but instead is via limbic forebrain structures, which are implicated in the sleep regulation. Furthermore, the low-frequency electrical stimulation of the medullary reticular formation, particularly the dorsal reticular formation and the caudal NTS, produces cortical synchronization indicative of slow-wave sleep (SWS) in an awake animal (14). Conversely, lesions of the dorsal reticular formation and of the NTS produced desynchronization of the EEG in a sleeping animal (15). All these results suggest the existence of neurons in the NTS that are involved in generating sleep. Furthermore, microinjection of morphine into the NTS provokes the enhancement of SWS and this effect is blocked by naloxone (16), suggesting the somnogenic effect of opioidergic system in the NTS. Our previous observations demonstrate that activation of cholinergic

system in the caudal NTS of the medulla oblongata mediated the enhancement of NREM sleep induced by EA stimulation of Anmian (EX17) acupoints (17). Nonetheless, EA may also increase β-endorphin concentrations in the NTS, which subsequently alter sleep, for the NTS area is one of the anatomically distinct β-endorphin pathways in the brain influenced by EA (18). Therefore, this study was designed to further clarify whether the activation of cholinergic system in the NTS after EA stimuli would enhance endogenous opioidergic activity and what type(s) of opioid receptors would be involved in EA-induced alterations in sleep.

There are two ascending pathways, NTS-VM projection and NTS-PBN-PVN/LHA projection. NTS and VM of the thalamus are well known participating in the regulation of sleep-wake homeostasis (19). However, the involvement of NTS-VM projections in mediating 10 Hz EA-induced sleep alteration remains unknown. VM receives opioidergic inputs from the NTS and the activation of opioid receptors hyperpolarizes neurons of VM (20). According to the projection pathway, we hypothesized that 10 Hz EA alters synaptic strength of the NTS by enhancement and changes the synaptic activity of the VM by downscaling, which contribute to the EA-induced NREM sleep enhancement. This hypothesis has been partially proven by that the EA-induced NREM sleep enhancement was blocked by muscarinic receptor antagonist in the NTS (18). We herein tried to further elucidate whether the changes of synaptic strength in both NTS

and VM are caused by morphological alterations of synaptic density and synaptic size.

The serial studies investigated the involvement of the NTS opioidergic system in EA-induced alterations in sleep, including: 1. Determining the opioid receptors and endogenous opiates in the NTS that involved sleep regulation after receiving high frequency (100 Hz) and low frequency (10 Hz) EA stimulation. 2. Investigating the synaptic morphology alteration in the NTS and the ventromedial nucleus (VM) of the thalamus where receives opioidergic inputs from the NTS. The following three chapters are the results of these studies. The first chapter is: the β-endorphin in the NTS mediated 10 Hz EA-induced sleep; the second: the κ-opioid receptors in NTS mediate 100 Hz EA-induced sleep, and the third part: the morphology alterations of synapse in the VM of thalamus and NTS after 10 Hz EA.

Neuropeptides, along with neurotransmitters, mediate various underlying mechanisms of neural functions and behaviors (e.g. opioid peptides in pain control (21), corticotrophin-releasing hormone (CRH) in stress-related behavior and sleep-wake regulation (22), hypocretin in feeding behavior and in the maintenance of vigilance states (23), and etc.). Discovery of endogenous opioid peptides, including β-endorphin, dynorphin, enkephalin and endomorphin, in the central nervous system (CNS) reveals the mysterious actions of acupuncture, especially in its analgesic effect. It had first been demonstrated that the acupuncture-induced analgesic effect could be blocked by a

broad-spectrum opioid receptor antagonist naloxone in both humans and mice (24,25), implicating the role of endogenous opioid peptides. Chang and Pomeranz had revealed that relatively low doses of naloxone only block the analgesic effect induced by low-frequency (4 Hz) of electroacupuncture (EA) stimulation, but not the consequence induced by high-frequency (200 Hz) of EA (26), suggesting that the low-frequency, rather than the high-frequency, of EA increases the release of endogenous opioids. Nevertheless, Han and his colleagues have further shown that the increase of endogenous opioids mediates the analgesic effects induced by both the low-frequency and high-frequency EA stimuli by employing distinct opioid receptor subtype-specific antagonists (27,28). While μ- and δ-opioid receptors in the spinal cord are dominant in the low-frequency EA-induced analgesia, κ-opioid receptors contribute to the high-frequency EA effects (27, 28). Radioimmunoassay of spinal perfusates from rats receiving various frequencies of EA stimulations further indicates that 2 Hz EA enhances enkephalin (a mixed μ- and δ-opioid receptor agonist) immunoreactivity (IR), 4 but not the dynorphin (κ-opioid receptor agonist) IR. In contrast, 100 Hz EA increases dynorphin IR rather than enkephalin IR (29).

Previous results have shown that 10 Hz EA at Anmian (EX17) acupoints increases slow wave sleep (SWS) in rats, which involves the induction of cholinergic activity in the caudal nucleus tractus solitaries (NTS) (17, 30). Our study has revealed that 10 Hz

(low frequency) EA stimulation of Anmian acupoints increases the concentrations of β-endorphin in the brainstem, which consequently enhances NREM sleep through activation of the μ-opioid receptors, rather than the δ- and κ-opioid receptors, in the caudal NTS (30). However, it has never been determined whether different frequencies of EA stimulations at Anmian acupoints activate distinct opioid receptors in the NTS. This study was also designed to clarify what type(s) of opioid receptor is (are) involved in high-frequency (100 Hz) EA-induced sleep alterations.

Chapter 2. Endogenous Opiates in the Nucleus Tractus Solitarius Mediate Electroacupuncture-induced Sleep Activities in Rats

2-1 Abstract

Electroacupuncture (EA) possesses various therapeutic effects, including alleviation of pain, reduction of inflammation and improvement of sleep disturbance. The mechanisms of EA on sleep improvement, however, remain to be determined. It has been stated in ancient Chinese literature that the Anmian (EX17) acupoint is one of the trigger points that alleviates insomnia. We previously demonstrated that EA stimulation of Anmian acupoints in rats during the dark period enhances non-rapid eye movement (NREM) sleep, which involves the induction of cholinergic activity in the nucleus tractus solitarius (NTS). In addition to cholinergic activation of the NTS, activation of the endogenous opioidergic system may also be a mechanism by which acupuncture affects sleep. Therefore, this study was designed to investigate the involvement of the NTS opioidergic system in EA-induced alterations in sleep. Our present results indicate that EA of Anmian acupoints increased NREM sleep, but not rapid eye movement sleep, during the dark period in rats. This enhancement in NREM sleep was dose-dependently blocked by microinjection of opioid receptor antagonist, naloxone, and the μ-opioid receptor antagonist, naloxonazine, into the NTS; administrations of δ-receptor antagonist, natrindole, and the κ-receptor antagonist, *nor*-binaltrophimine, however, did not affect

EA-induced alterations in sleep. Furthermore, β-endorphin was significantly increased in both the brainstem and hippocampus after the EA stimuli, an effect blocked by administration of the muscarinic antagonist scopolamine into the NTS. Our findings suggest that mechanisms of EA-induced NREM sleep enhancement may be mediated, in part, by cholinergic activation, stimulation of the opiodergic neurons to increase the concentrations of β-endorphin and the involvement of the μ-opioid receptors.

2-2 Introduction

Electroacupuncture (EA), which consists of passing a continuous electric current through needles inserted into the acupoints to obtain the therapeutic effects, i.e. alleviation of pain, reduction of inflammation and management of insomnia, is modified from the traditional Chinese acupuncture. Insomnia is one of the most common sleep disorders and it has been demonstrated that the effectiveness rate of acupuncture for relieving insomnia is about 90% (2,3). Several specific acupoints have been identified for insomnia treatment based upon the differentiation and signs of symptoms according to traditional Chinese medicine. Among the acupoints used, Shenmen (HT7), Sanyinjiao (SP6) and Anmian (EX17) are the most common, although other acupoints may also be used, such as Neiguan (PC6), Zusanli (ST36), Taichong (LR3), Baihui (DU20), Dazhui (DU40), Tainzhu (BL10), Bishu (BL20) and Zhongwan (RN12) (4,5).

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limbic forebrain structures, which are implicated in the sleep regulation. Furthermore, the low-frequency electrical stimulation of the medullary reticular formation, particularly the dorsal reticular formation and the caudal NTS, produces cortical synchronization indicative of slow-wave sleep (SWS) in an awake animal (14). Conversely, lesions of the dorsal reticular formation and of the NTS produced desynchronization of the EEG in a sleeping animal (15). These results all suggest the existence of neurons in the NTS that are involved in generating sleep. Furthermore, microinjection of morphine into the NTS provokes an enhancement of SWS and this effect is blocked by naloxone (16), suggesting the somnogenic effect of opioidergic system in the NTS. Our previous observations demonstrate that activation of cholinergic system in the caudal NTS of the medulla oblongata mediates the enhancement of non-rapid eye movement (NREM) sleep induced by EA stimulation of Anmian (EX17) acupoints (17). Nonetheless, EA may also increase β-endorphin concentrations in the NTS, which subsequently alter sleep, because the NTS area is one of the anatomically distinct β-endorphin pathways in the brain influenced by EA (18). Therefore, this study was designed to further clarify whether the activation of cholinergic system in the NTS after EA stimuli enhances endogenous opioidergic activity and what type(s) of opioid receptors are involved in EA-induced alterations in sleep.

2-3 Methods

2-3-1 Pharmacological agents

Stock solutions of muscarinic receptor antagonist, scopolamine hydrobromide (Sigma, St Louis, MO, USA), a broad-spectrum opioid antagonist [naloxone hydrochloride (Tocris, Bristol, UK)], a μ-receptor antagonist [naloxonazine dihydrochloride (Tocris)], a δ-receptor antagonist [naltrindole hydrochloride (Tocris)] and a κ-receptor antagonist [nor-binaltorphimine dihydrochloride (Tocris)] were dissolved in pyrogen-free saline (PFS). The stock solutions were stored at 4℃ until use. The dose of scopolamine used was 20 μg μl^{-1}, whereas naloxone, naloxonazine, naltrindole and *nor*-binaltorphimine were microinjected at three different doses, 0.1, 1.0 and 10 μg μl^{-1}. The total volume used for each microinjection was 1 μl.

2-3-2 Animals

Male Sprague-Dawley rats (250 ~ 300 g; National Laboratory Animal Breeding and Research Center, Taiwan) were used in this study. Rats were anesthetized by intraperitoneal injection with ketamine/xylazine (87/13 mg kg^{-1}) and were given an analgesic (1 mg/rat morphine) and an antibiotic (5000 IU/rat penicillin G benzathine) to reduce pain and avoid infection. Rats were surgically implanted with three electroencephalogram (EEG) screw electrodes as earlier described (31) and the

microinjection guide cannulae directed into the NTS (AP, 13.30mm from bregma; ML, 1.2mm and DV, 8.2mm relative to bregma). The coordinates were adopted from the Paxinos and Waton rat altas (32). Two unilateral screw EEG electrodes were placed over the right hemisphere of the frontal and parietal cortices and a third EEG electrode was placed over the cerebellum and served to ground the animal to reduce signal artifacts. 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 7 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 ℃ and the light:dark rhythm was controlled in a 12 : 12 h cycle (40 Watt × 4 tubes illumination). Food (5001 rodent diet, LabDiet) and water were available ad libitum. All procedures performed in this study were approved by the National Taiwan University Animal Care and Use Committee.

2-3-3 Experimental Protocol

On the second postsurgical day, the rats were connected to the recording apparatus via a flexible tether. As such, the rats were allowed relatively unrestricted movement within

their own cages. Three groups of rats were used in the study as follows: Group 1 (*n=8*) was used to determine the effects of opioid receptor antagonist (naloxone) and μ-receptor antagonist (naloxonazine) on EA-induced alterations in sleep; Group 2 (*n=8*) was used to elucidate the effects of δ-receptor antagonist (naltrindole) and κ-receptor antagonist (*nor*-binaltorphimine) on EA-induced alterations in sleep; Group 3 (*n=24*) was used to depict the action of NTS muscarinic receptors on EA-induced β-endorphin expression [determined by enzyme-linked immunosorbent assay (ELISA)] after microinjection of scopolamine into the NTS. One week after rats had adapted to the 12 : 12 h light : dark cycle after surgery, a 23-h undisturbed baseline recordings were obtained beginning at dark onset on the first recording day in rats in Groups 1 and 2. When EA was given (see later), all rats were lightly anesthetized with one-third of the dose of ketamine/xylazine used in the surgery, after which rat wake-up time is 20 ~ 25 min. A 20-min period of EA stimulation was administered before the onset of the dark period. The anesthetization was given 25 min prior to the dark period onset and lasted for 20 min. The rationale for carrying out the experiment in the darkness is that rats are active with a lowest level of sleep during the dark period, and a manipulation, if it possesses ability to increase sleep, would significantly augment sleep during the dark period. In contrast, it may not be easy to enhance sleep during the light period when sleep activity is at its highest circadian level. Since we expected to find a sleep enhancement after the EA stimuli at the Anmian (EX17),

we therefore manipulated the EA stimulation before the onset of the dark period and analyzed the sleep alteration during the subsequent dark period. The rats in group 1 were both administered PFS intraperitoneally (IP) and microinjected into the NTS ($_{ip}$PFS+PFS) at 25 min prior to the dark onset on two consecutive days, and recordings obtained for 24-h beginning after the second injection. The effects of anesthesia with the NTS microinjection of PFS ($_{ip}$ketamine+PFS) on sleep were determined after IP injection of ketamine/xylazine and the NTS PFS microinjection on two consecutive days. A sham EA ($_{ip}$ketamine+PFS+sham EA) was delivered to control for the non-specific effect of the electrical stimulation, although our previous study had confirmed that no non-specific effect was observed after the sham EA (17). The EA stimuli under anesthesia ($_{ip}$ketamine+PFS+EA) were also performed before the dark onset on two consecutive days and sleep-wake behavior after the second EA stimulation was then determined. Subsequently, three different doses (0.1, 1.0 and 10 μg) of naloxone ($_{ip}$ketamine+ naloxone+EA) and naloxonazine ($_{ip}$ketamine+naloxonazine+EA) were administered 25 min prior to the dark onset on the second day of EA stimulation and the 24-h sleep pattern was determined. At least 1 day without injections was scheduled between each manipulation. The EA stimulus was delivered via the bilateral insertion of stainless needles (32 gauge×1", Shanghai Yanglong Medical Articles Co.) on Anmian (EX17) points in the depth of 2 mm. The stimulus consisted of a train of biphasic pulses (150 μs

duration each) of 10 Hz with intensity of 3 mA, and was delivered by Functions Electrical Stimulator (Trio 300, I.T.O., Japan). The acupoint 'Anmian (EX17)' is located at midpoint between Yifeng (TH 17) and Fengchi (GB 20); Yifeng (TH 17) locates posterior to the lobule of the ear in the depression between the mandible and mastoid process and Fengchi (GB 20) locates in the depression between the upper portion of musculus sternocleidomastoideus and musculus trapezius in human. The location of Anmian (EX17) in rats is at the relative anatomical location between the strenocleidomastoideus muscle and the splenius capitis muscle, as in the human acupoint map. Sham EA was performed by stimulation of a non-acupoint located at the ventral conjunction between the forelimb and the trunk as described earlier (17). Those rats in group 2 underwent a similar protocol to those in group 1, except that the substances administered were naltrindole ($_{ip}$ketamine+naltrindole+EA) and *nor*-binaltorphimine ($_{ip}$ketamine+ *nor*-binaltorphimine+EA). Rats in group 3 were divided into four subgroups and received $_{ip}$ketamine+PFS, $_{ip}$ketamine+PFS+sham EA, $_{ip}$ketamine+PFS+EA and $_{ip}$ketamine+ scopolamine+EA, respectively. Microinjection of scopolamine into the NTS was administered prior to the second EA stimulation. Rats were then decapitated at the dark onset and five distinct brain regions, including the hypothalamus, cortex, brainstem, hippocampus and striatum, were dissected and frozen in -80℃ until assay. These tissues were used for β-endorphin ELISA as described in the following.

2-3-4 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 5000) and 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 analogto-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.

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 SWS, rapid eye movement sleep (REMS) or waking based on previously defined criteria (31). Briefly, SWS is characterized by large-amplitude EEG slow waves, high power density values in the delta frequency band (0.5 ~ 4 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 ~ 90 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. In addition to the amount of time spent in each vigilance state, the number and duration of individual bouts of behaviors were determined using criteria modified from those of Tobler and colleagues (33,34), as described earlier (31).

2-3-5 ELISA for β-endorphin

Rat β-endorphin ELISA kits were obtained from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA) and the procedures followed the standard instructions provided by the manufacturers. Absorbance was measured by an ELISA plate reader (Multiskan EX, Thermo Electron Corp., Waltham, MA, USA) with the wavelength set at 450 and 550 nm. The sensitivity is 0.21 ng ml^{-1}, the assay range is between 0.21 and 3.15 ng ml^{-1}, the intra-assay coefficient of variation is <5% and the inter-assay coefficient of variation is <14%. There is no cross-reactivity for met-enkephalin and leu-enkephalin.

2-3-6 Statistical Analyses for Experiment Protocol

All values acquired from sleep-wake recording 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, comparing before and after manipulation within subjects, across a certain of time block. An a level of $P \leqq 0.05$ was taken as indicating a statistically significant difference. If statistically significant differences were detected, a *Scheffe post hoc* comparison was made to determine which hourly intervals during experimental conditions deviated from values obtained from the same animals during control conditions. The statistical evaluation for the β-endorphin ELISA used an unpaired Student's *t*-test comparing the averages between the groups analyzed.

2-4 Results

2-4-1 Naloxone and Naloxonazine Blocked the EA-induced Alterations in Sleep

Anesthetization of rats for 25 min with ketamine/xylazine prior to the dark period suppressed both NREM and REM sleep for the first 4 h of the dark period. The time spent in NREM sleep during the first 4-h period after $_{ip}$ketamine+PFS decreased to 10.8±2.5% from 22.4±2.5% acquired after $_{ip}$PFS+PFS [$F_{(1,62)}$=10.711, P=0.002, Fig. 1A and B]. REM sleep was suppressed from 6.5±1.8% obtained after $_{ip}$PFS+PFS to 0.8±0.3% acquired after $_{ip}$ketamine+PFS [$F_{(1,62)}$=8.697, P=0.005, Fig. 1C and D]. A concomitant enhancement of wakefulness was observed during the first 4 h after

receiving ketamine anesthetization (Fig. 1E and F). Application of sham EA did not alter any aspect of sleep parameters (data not shown), which is similar to our previous observation (17). Twenty-minute EA stimuli delivered before the dark period on two consecutive days significantly augmented NREM sleep during post-manipulation 5 ~ 8 h. The percentage of time spent in NREM sleep during 5 ~ 8 h increased from 18.3±2.7% obtained after $_{ip}$ketamine+PFS to 34.0±3.8% after $_{ip}$ketamine+PFS+EA [$F_{(1,62)}$=9.180, P=0.004, Fig. 1A and B]. Analysis of 12-h dark period revealed that NREM sleep was enhanced from 14.8±1.5% after $_{ip}$ketamine+PFS to 21.7±2.1% after $_{ip}$ketamine+PFS+EA [$F_{(1, 190)}$=6.923, P=0.010]. REM sleep was not significantly altered by EA. A decrease of waking at the expense of SWS was observed during 5 ~ 8 h (Fig. 1E and F).

Administration of three different doses (0.1, 1.0 and 10.0 μg) of naloxone, a broad-spectrum opioid antagonist, into the NTS dose-dependently blocked EA-induced increases of NREM sleep, especially during post-manipulation 5 ~ 8 h (Fig. 2A and B). Across the entire 12-h dark period recording, NREM sleep was suppressed from 21.7±2.1% after $_{ip}$ketamine+PFS+EA to 13.4±2.1% after $_{ip}$ketamine+naloxone (10.0 μg)+EA [$F_{(1,190)}$=7.631, P=0.007, Fig. 2A and B]. Administration of μ-opioid receptor antagonist, naloxonazine, also exhibited a dose-dependent blockade of EA-inducedNREM sleep enhancement (Fig. 2A and B). Administration of 10 μg

naloxonazine reduced NREM sleep from 21.7±2.1% after $_{ip}$ketamine+PFS+EA to 14.9±2.1% [$F_{(1,190)}$=5.100, P=0.026, Fig. 2A and B] during the 12-h dark period. Neither naloxone nor naloxonazine altered REM sleep (Fig. 2C and D). A mirror effect on the EA-induced decrease of waking was also been observed for both naloxone and naloxonazine (Fig. 2E and F).

Analysis of sleep architecture parameters across 1 ~ 12 h during the dark period revealed that the effect of ketamine on the suppression of NREM and REM sleep was primarily due to an increase in episode duration of waking, although there was a tendency for decreases in both REM sleep episode number and in NREM sleep episode duration (Table 1). The increase of NREM sleep after EA stimuli was primarily because of the increase in the duration of a single episode (Table 1), which is similar to our previous result (17). Effects ofnaloxone and naloxonazine on blocking EA-induced enhancement of NREM sleep were mediated by reversing the EA-induced augmentation of NREM sleep episode duration (Table 1).

2-4-2 Naltrindole and *Nor*-binaltorphimine did not Affect EA-induced Alterations in Sleep

Administration of either the δ-opioid receptor antagonist naltrindole or the κ-receptor antagonist *nor*-binaltorphimine prior to EA stimulation did not affect EA-induced

alterations in sleep (Fig. 3). Similarly, these antagonists did not alter any aspect of sleep architecture (data not shown).

2-4-3 Scopolamine Suppressed EA-induced Expression of Endogenous β-endorphin

Our result demonstrated that delivery of sham EA stimuli did not alter the concentrations of β-endorphin within five distinct brain structures, including the brainstem, hypothalamus, cortex, hippocampus and striatum (Fig. 4). However, application of EA stimuli at Anmian (EX17) acupoints for 20 min significantly enhanced the levels of β-endorphin in the brainstem and hippocampus, but not in the cortex, hypothalamus and striatum (Fig. 4). Administration of the muscarinic receptor antagonist scopolamine directly into the NTS before the EA stimuli blocked EA-induced increases of β-endorphin in the brainstem and hippocampus (Fig. 4).

Figure 1. The effects of low-frequency (10 Hz) stimulation by EA at Anmian (EX17) acupoints on vigilance states. The 10 Hz EA enhanced NREM sleep during post-manipulation 5 ~ 8 h during the subsequent dark period. *Represents a statistically significant difference between the values obtained from ipketamine+PFS and ipPFS+PFS. The shaded area represents the values of mean±SEM. #Depicts a statistically significant difference between the values obtained from ipketamine+PFS+EA and ipketamine+PFS. The black horizontal bar on the x-axis represents the dark period of the 12 : 12 h light : dark cycle. WAKE, wakefulness.

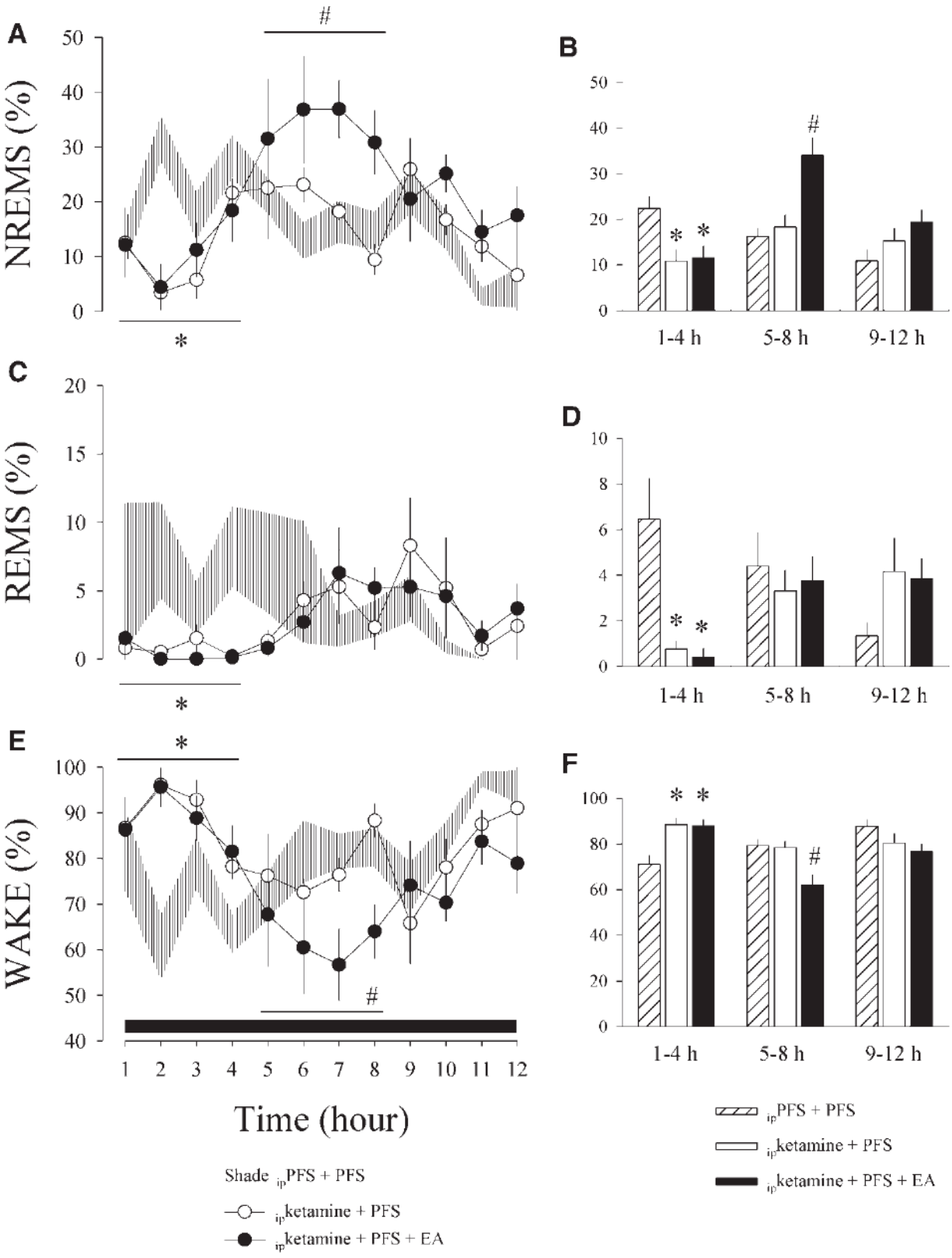

A
NREMS (%)
B
C
REMS (%)
D
E
WAKE (%)
F
1-4 h
5-8 h
9-12 h
Time (hour)
Shade ip PFS + PFS
ip ketamine + PFS
ip ketamine + PFS + EA

Figure 2. The effects of naloxone and naloxonazine on EA-induced alterations in sleep. Both naloxone and naloxonazine dose-dependently blocked the EA-induced enhancement of NREM sleep. *Represents a statistically significant difference between the values obtained from $_{ip}$ketamine+naloxone+EA/$_{ip}$ketamine+ naloxonazine+EA and $_{ip}$ketamine+PFS+EA. The black horizontal bar on the x-axis represent the dark period of the 12 : 12 h light : dark cycle.

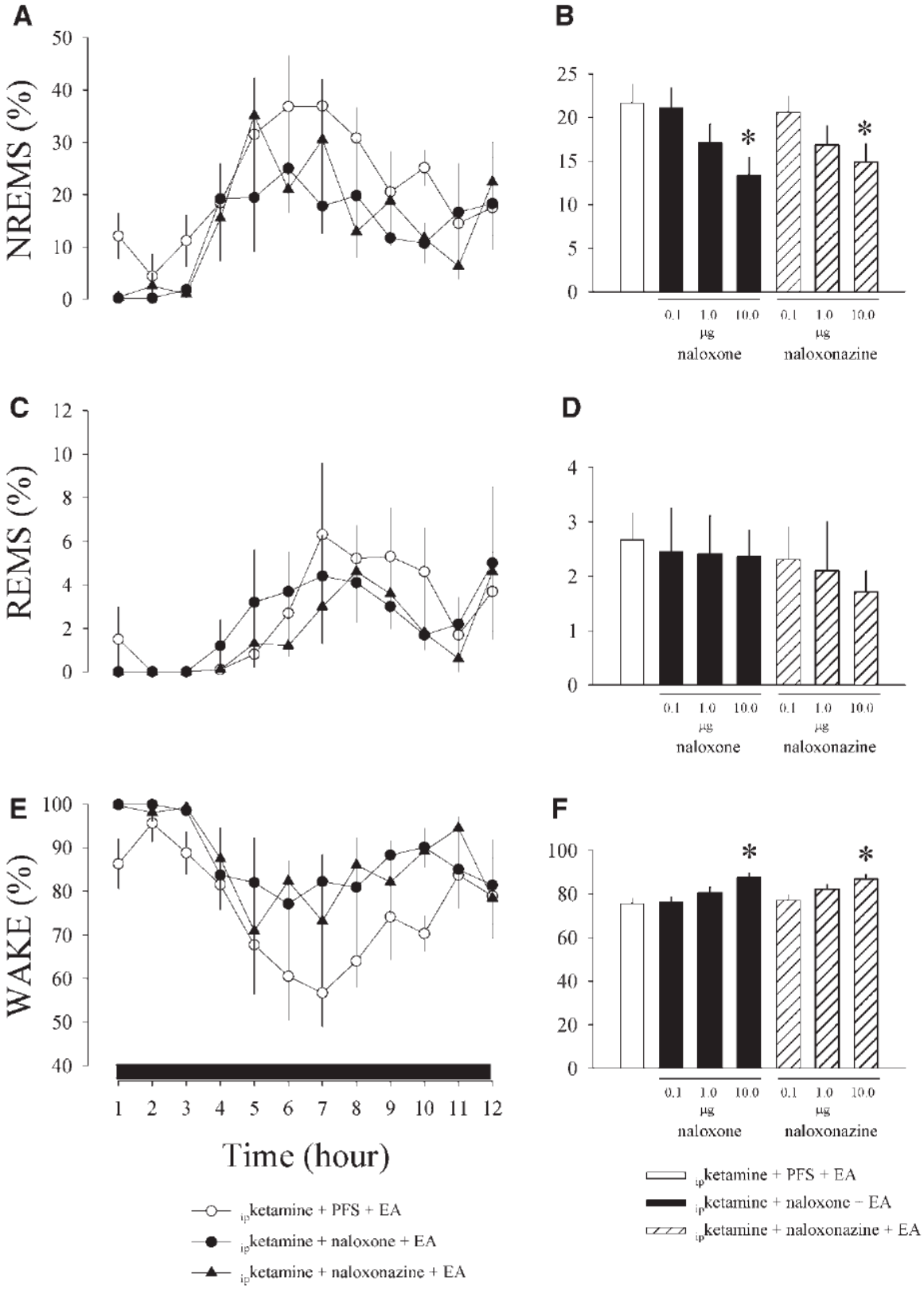

A
NREMS (%)
B
C
REMS (%)
D
E
WAKE (%)
Time (hour)
F
0.1
1.0
10.0
μg
naloxone
naloxonazine
ipketamine + PFS + EA
ipketamine + naloxone + EA
ipketamine + naloxonazine + EA

Figure 3. The effects of naltrindole and *nor*-binaltorphimine on EA-induced alterations in sleep. Neither naltrindole nor *nor*-binaltorphimine affected EA-induced enhancement of NREM sleep. * Represents a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS+EA/ $_{ip}$ketamine+natrindole+EA/ $_{ip}$ketamine+ *nor*-binaltorphimine+EA and $_{ip}$ketamine+PFS. The shaded area represents the values of mean±SEM. The black horizontal bar on the x-axis represents the dark period of the 12 : 12 h light : dark cycle.

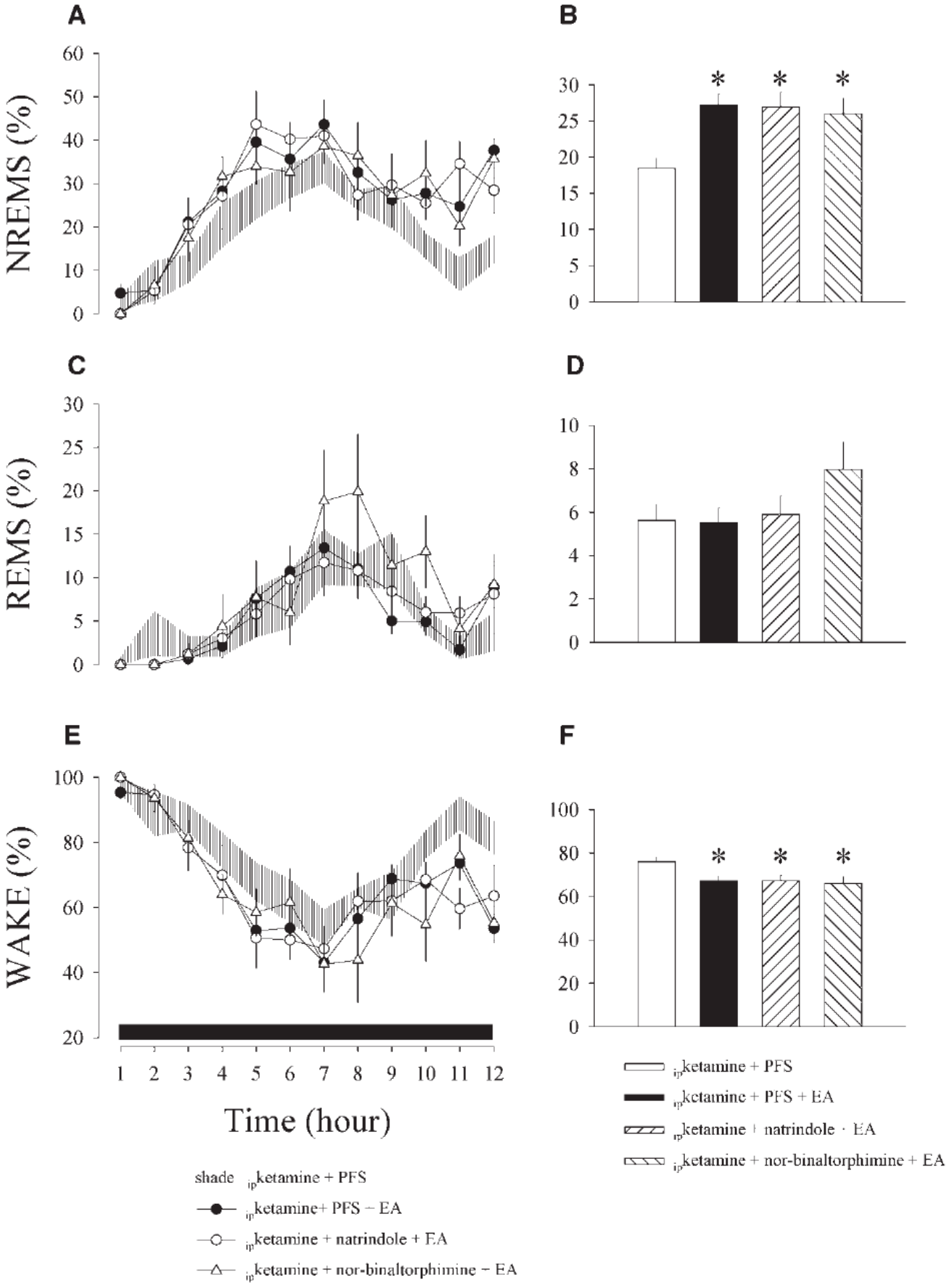

A
B
C
D
E
F
NREMS (%)
REMS (%)
WAKE (%)
Time (hour)
shade ipketamine + PFS
ipketamine+ PFS − EA
ipketamine + natrindole + EA
ipketamine + nor-binaltorphimine − EA
ipketamine + PFS
ipketamine + PFS + EA
ipketamine + natrindole + EA
ipketamine + nor-binaltorphimine + EA

Figure 4. The effects of scopolamine on the 10 Hz EA stimuli-induced increase of β-endorphin. The 10 Hz EA stimuli significantly increased the expressions of β-endorphin in the brainstem and hippocampus and administration of scopolamine blocked EA-induced increases of β-endorphin concentration. *Represents a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS+EA and $_{ip}$ketamine+PFS+sham EA. #Depicts a statistically significant difference between the values obtained from $_{ip}$ketamine+scopolamine+EA and $_{ip}$ketamine+PFS+EA.

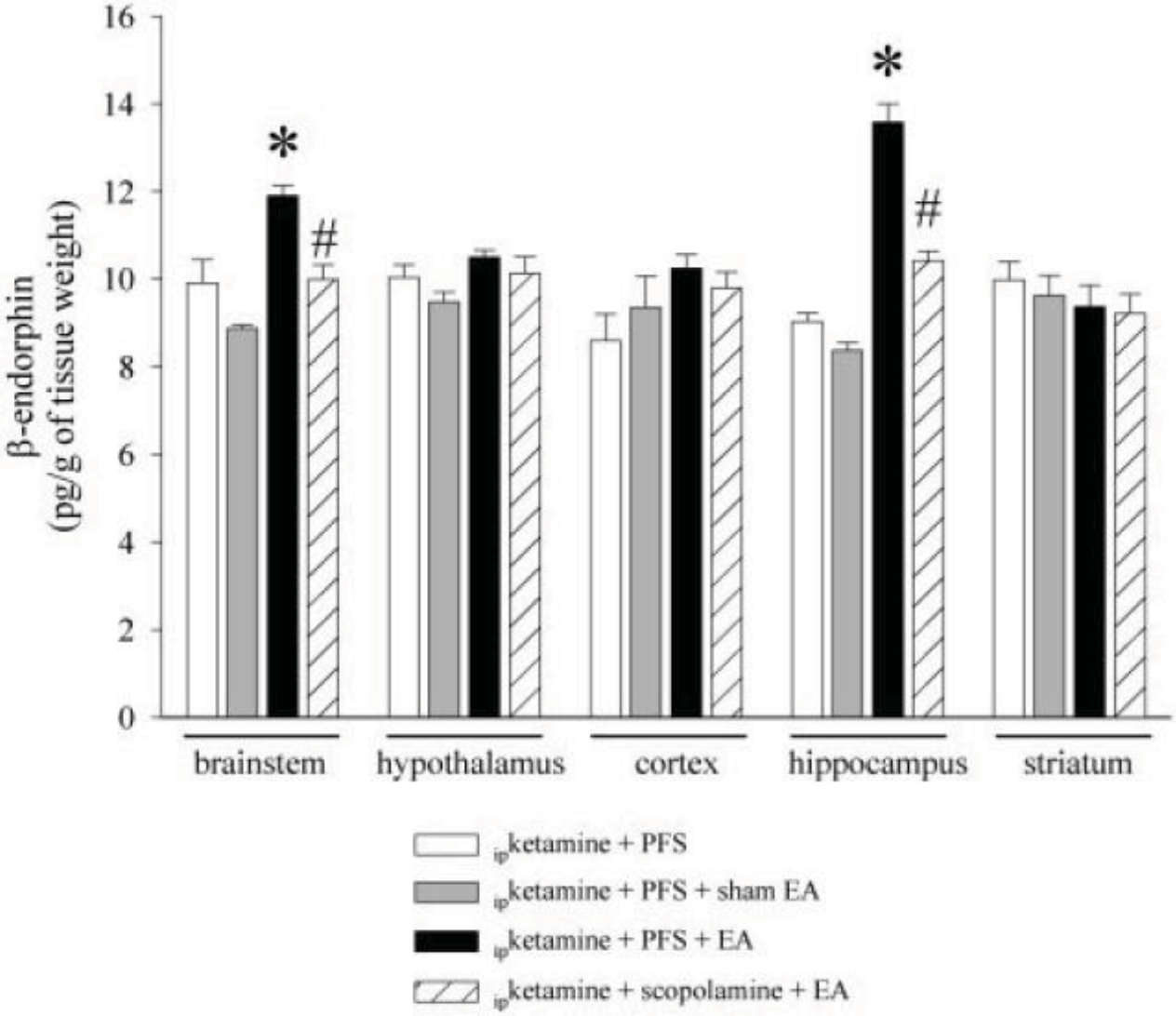

β-endorphin
(pg/g of tissue weight)
16
14
12
10
8
6
4
2
0
*
#
*
#
brainstem
hypothalamus
cortex
hippocampus
striatum
ipketamine + PFS
ipketamine + PFS + sham EA
ipketamine + PFS + EA
ipketamine + scopolamine + EA

Figure 5. A hypothetical model by which EA Anmian (EX17) may alter NREM sleep. EA stimuli in the figure represents the electrical sign; Ach: acetylcholine; electrical sign: EA stimuli.

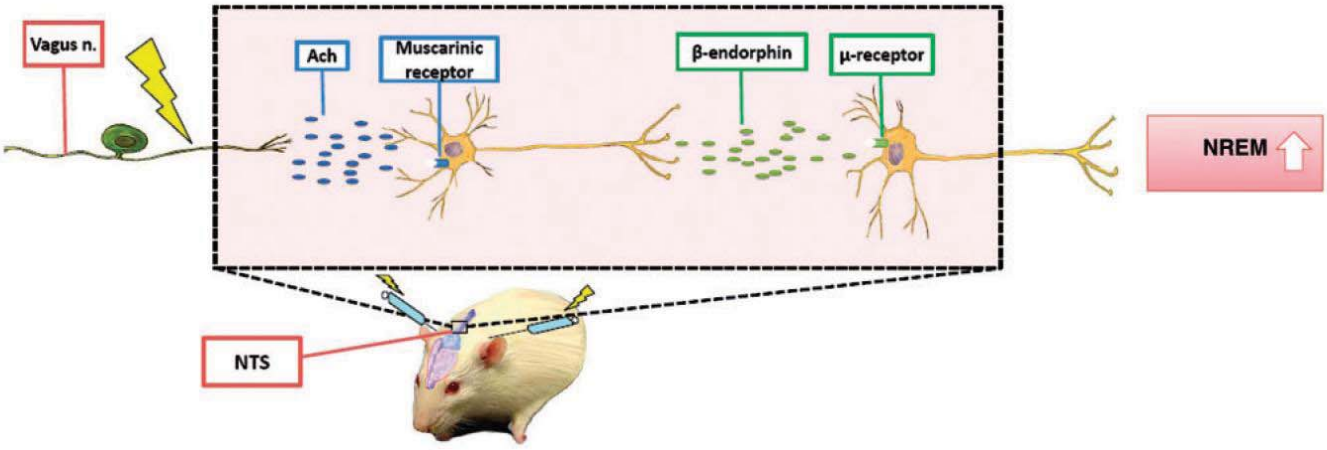

Vagus n.
Ach
Muscarinic receptor
β-endorphin
μ-receptor
NREM
NTS

Table 1. Effects of naloxone and naloxonazine on the alterations of sleep–wake architecture parameters induced by EAc of Anmain (EX17) acupoints in rats.

Manipulation[d]	Hour	L:D cycle[e]	Number of bouts[a]			Bout duration[b]			Transitions[c]
			WAKE[f]	NREMS[f]	REMS[f]	WAKE	NREMS	REMS	
$_{ip}$PFS + PFS	1–12	D	3.98 ± 0.31	5.06 ± 0.45	1.58 ± 0.26	18.54 ± 2.20	1.67 ± 0.15	0.67 ± 0.10	26.94 ± 2.31
$_{ip}$Ketamine + PFS	1–12	D	3.52 ± 0.36	4.40 ± 0.46	1.20 ± 0.24	24.12 ± 2.97*	1.44 ± 0.15	0.59 ± 0.10	25.93 ± 2.62
$_{ip}$Ketamine + PFS + EAc	1–12	D	3.43 ± 0.30	4.75 ± 0.46	1.38 ± 0.24	20.57 ± 2.65	2.13 ± 0.20$^{\#}$	0.54 ± 0.09	28.81 ± 2.60
$_{ip}$Ketamine + naloxone + EAc	1–12	D	3.20 ± 0.44	3.48 ± 0.58	1.21 ± 0.26	29.17 ± 3.53$^{\$}$	1.15 ± 0.20$^{\$}$	0.66 ± 0.11	28.22 ± 3.51
$_{ip}$Ketamine + naloxonazine + EAc	1–12	D	3.19 ± 0.43	4.27 ± 0.65	0.98 ± 0.21$^{\$}$	26.16 ± 3.30	1.26 ± 0.17$^{\$}$	0.48 ± 0.10	27.93 ± 3.53

Values are means ± SEM.
[a]Number of bouts per hour (mean ± SEM) for each vigilance state; [b]Mean (±SEM) bout duration (min) for each vigilance state; [c]Number of transitions from one behavioral state to another (mean ± SEM) per hour; [d]Experimental manipulation; [e]Period of the light:dark cycle immediately prior to which injections were given: D = dark period; [f]Vigilance states: WAKE, wakefulness; NREMS, non-rapid eye movement sleep; REMS, rapid eye movements sleep; *Denotes a statistically significant difference ($P < 0.05$) between values obtained after administration of $_{ip}$PFS + PFS and those obtained after receiving $_{ip}$ketamine + PFS; $^{\#}$Depicts a statistically significant difference ($P < 0.05$) between values obtained after $_{ip}$ketamine + PFS + EAc and those obtained after $_{ip}$ketamine + PFS; $^{\$}$Depicts a statistically significant difference ($P < 0.05$) after either receiving naloxone or naloxonazine.

2-5 Discussion

Insomnia is a common sleep complaint among elderly people and young adults, which may result from psychiatric illness, sociopsychological stress, a medical problem, poor sleep habits or a primary sleep disorder. Epidemiological surveys have shown that 10 ~ 20% of adults have suffered from moderate to severe insomnia (35), although the percentage is lower than that of 40 ~ 70% of healthy elderly people who suffer from chronic sleep disturbances (36). Sedative-hypnotic medications, including benzodiazepines and non-benzodiazepines, are the most common treatments for insomnia. However, there are concerns regarding the inappropriate use, dependence and adverse effects of these agents. On the other hand, acupuncture has been used for relieving sleep disturbances for thousands of years in China. Although acupuncture is efficacious for sleep problems, especially for insomnia, the underlying mechanisms whereby sleep is improved by acupuncture are poorly understood. We, therefore, designed this study to determine whether the opioidergic system of the NTS plays a role in EA-induced alterations in sleep. Our current results demonstrate that EA stimuli of Anmian (EX17) acupoints in anesthetized rats for 20 min enhances NREM sleep during the subsequent dark (active) period. This observation is a further confirmation of the ability of EA stimuli at Anmian (EX17) acupoints to increase NREM sleep. In order to perform the EA stimulation easily, rats were lightly anesthetized. We found that both NREM and REM

sleep during the first 4h of the dark period were decreased after rats recovered from the ketamine anesthetic. Ketamine, a cyclohexanone derivative, is used clinically as a dissociative anesthetic agent both in humans and animals. Ketamine is a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist that blocks cation channels (37). It has been demonstrated that administration of ketamine or MK-801, another NMDA receptor antagonist, at sub-anesthetic doses produce a robust, dosedependent increase in the intensity of δ-power of the NREM sleep (38,39). Furthermore, the effect of MK-801 by increasing the metabolic rate in the hippocampus and other limbic structures stimulates physiological sleep that is similar to the sleep that follows sleep deprivation, indicating the need of homeostatic recovery (40). Therefore, the suppression of NREM and REM sleep after recovery from the ketamine anesthetization during the beginning of the dark period may be due to a homeostatic compensation to the previous anesthetic state. However, this explanation needs to be further investigated. On the other hand, EA of other acupoints may exhibit an opposite effect on sleep or anesthesia. For example, EA stimulation of bilateral Neiguan (PC6) shortened the post-anesthesia recovery time in cats (41). However, EA of Anmian (EX17) did not exhibit this effect in our experiment.

We previously demonstrated that microinjections of muscarinic receptor antagonist scopolamine into the NTS and bilateral lesions of the caudal NTS blocks the alterations in sleep induced by EA stimulation of Anmian acupoints (17), implicating the involvement

of cholinergic neurons in the caudal NTS in this response. Nevertheless, endogenous opiates (β-endorphin, enkepalin, endomorphin and dynorphin) are well known to mediate the analgesic effect of EA; low frequency EA stimulation activates μ- and δ-opioid receptors by the releases of β-endorphin, enkepalin and endomorphin, whereas high-frequency EA stimulation activates the κ-receptors by enhancing the concentrations of dynorphin (21,42). It has also been demonstrated that EA increases β-endorphin concentrations in the arcuate nucleus of the hypothalamus via Aβ and Aδ fibers, which in turn mediate the analgesic effects of EA (43).

There are two anatomically distinct β-endorphin pathways in the brain; the major pathway originates in the arcuate nucleus and the minor one is in the area of the NTS of the caudal medulla (18). Therefore, it is possibility that β-endorphin concentrations may increase in the caudal NTS after the EA stimulation. Reinoso-Barbero and de Andres have shown that microinjection of morphine into the NTS provokes a dose-dependent enhancement of the polygraphic and behavioral manifestations of SWS. This effect is blocked by naloxone, suggesting that the endogenous opioid is involved in controlling electrocortical activity generated by the NTS (16). Furthermore, it has been documented that vagal afferents activate the β-endorphin system in the NTS, which in turn mediates responses to vagal activation; the effects of acetylcholine on depressive responses in pulmonary and carotid arteries are mediated by activating the serotonergic and endorphin

system of the NTS (44) and the inhibitory effect of vagal afferents on the bradycardia response is mediated by β-endorphinergic neurons in the NTS (9). On the basis of our previous results (17) and aforementioned evidence, we herein tried to elucidate whether the effects of EA on sleep regulation is mediated by the increase of endogenous opiates and the activation of opioidergic receptors in the caudal NTS via muscarinic receptors. Application of naloxone, a broad spectrum of opioid receptor antagonist, was used to determine the involvement of NTS opioidergic receptors in the EA-induced increase of NREM sleep. We found that administration of naloxone directly into the caudal NTS dose-dependently blocked EA-induced NREM sleep enhancement during the dark period, but had no effect on the REM sleep, implicating the involvement of endogenous opiates in the NTS. A potential role for three major opioid receptors, μ-, δ- and κ-receptors, was then determined by application of specific receptor antagonists. Our results demonstrate that naloxonazine, a μ-opioid receptor antagonist, exhibits a similar dose-dependent effect as that of naloxone, whereas naltrindole (a δ-opioid receptor antagonist) and *nor*-binaltorphimine (a κ-opioid receptor antagonist) have no effect. These results are in part consistent with the results reported by Reinoso-Barbero and de Andres that administration of μ-and δ-opioid receptor agonists, but not κ-opioid receptor agonist, into the NTS in cats enhances NREM sleep and that REM sleep is unchanged after NTS administration of the different opioids (16). Nevertheless, our results implicate NTS

μ-opioid receptors as mediators of EA-induced NREM sleep enhancement, which is similar to the effect of μ-opioid receptors on analgesia induced by low-frequency EA stimuli. The frequency of EA we used in this study was 10 Hz, which is considered a low-frequency stimulation. Our results also indicate that concentrations of β-endorphin in the brainstem and hippocampus increase after EA stimuli, suggesting the involvement of NTS β-endorphin. The enhancement of β-endorphin in the hippocampus may be due to actions of multisynaptic relays from the NTS to the hippocampus (45). Enkephalin- and β-endorphin-containing neurons are in the NTS (46). Therefore, the expression of enkephalin and endorphin after low-frequency EA stimuli and the expression of dynorphin after high-frequency EA would be of interest for further investigation. Nevertheless, EA-enhanced β-endorphin in the NTS is blocked by microinjection of muscarinic receptor antagonist scopolamine, suggesting that the activation of endogenous opioidergic system is mediated by the NTS cholinergic nervous system.

2-6 Conclusion

In summary, our current results demonstrate that EA stimuli of Anmian (EX17) acupoints enhance NREM sleep and this enhancement is blocked by naloxone and naloxonazine, implicating μ-opioid receptors. Furthermore, the activation of NTS muscrainic receptors after low-frequency EA stimuli increases concentrations of

β-endorphin, which mediates the enhancement of NREM sleep after EA stimuli of Anmian (EX17) acupoints. A diagram elucidating one hypothetical mechanism by which EA of Anmian (EX17) alters sleep is depicted in Fig. 5.

Chapter 3. Morphology Alterations of Synapses in the Ventromedial Nucleus (VM) of Thalamus and Caudal Nucleus Tractus Solitarius (NTS) after 10 Hz Electroacupuncture in Rats

3-1 Abstract

Previous results demonstrated that 10 Hz electroacupuncture (EA) of Anmian acupoints in rats during the dark period enhances slow wave sleep (SWS), which involves the induction of cholinergic activity in the caudal nucleus tractus solitarius (NTS) and subsequent activation of opioidergic neurons and μ-receptors. One ascending projection is from NTS to the ventromedial nucleus (VM) of the thalamus (the NTS-VM pathway). Wakefulness is accompanied by synaptic potentiation in the cortical circuits, whereas slow wave activity (SWA) during SWS promotes a generalized depression or downscaling of synaptic strength. The VM receives opioidergic inputs from NTS and the activation of opioid receptors hyperpolarize neurons of VM. Accordingly, 10 Hz EA may increase synaptic activity of NTS and subsequently hyperpolarize and downscale the synaptic strength in the VM of thalamus by inhibitory afferents, which lead to enhance SWS. Enhancement of excitatory synapses in NTS and inhibitory synapses in VM may respectively contribute to the up-regulation of synaptic strength in NTS and downscaling of synaptic strength in the VM after 10 Hz EA. Our results demonstrated that the synaptic density was increased in both NTS and VM after

rats received 10 Hz EA stimuli, while the enhanced synaptic length was only observed in the NTS, suggesting that 10 Hz EA altered excitatory synaptic strength of NTS and inhibitory synaptic strength of VM by changing the synaptic morphology.

3-2 Introduction

Electroacupuncture (EA), which consists of passing a continuous electric current through needles inserted into the acupoints to obtain therapeutic effects, i.e. alleviation of pain, reduction of inflammation and management of insomnia, is modified from the traditional Chinese acupuncture. Insomnia is one of the most common sleep disorders. It has been demonstrated that the effectiveness rate of acupuncture for relieving insomnia is about 90% (2,3). Several specific acupoints have been identified for insomnia treatment based upon the differentiation and signs of symptoms according to traditional Chinese medicine. Among the acupoints used, Shenmen (HT7), Sanyinjiao (SP6) and Anmian (EX17) are the most common, although other acupoints may also be used, such as Neiguan (PC6), Zusanli (ST36), Taichong (LR3), Baihui (DU20), Dazhui (DU40), Tainzhu (BL10), Bishu (BL20) and Zhongwan (RN12) (4, 5).

The mechanisms by which EA functions to alleviate clinical symptoms remain largely unknown, although applications of EA have been widely described in the Chinese literature (1,6,7,8). Recent findings suggest that the induction of vagus nerve

activity appears to be a significant factor for mediating the action of acupuncture (9, 10). The caudal nucleus tractus solitarius (NTS) may be activated by acupuncture, since NTS is located in the dorsomedial medulla oblongata and receives afferents primarily from the vagus and glossopharyngeal nerves (11). Ascending projections from the NTS are traced through the lateral and dorsal tegmentum and periventricular gray up to the rostral pons and midbrain and terminate in the parabrachial nucleus (PBN), ventromedial nucleus (VM) of thalamus, paraventricular nucleus (PVN) of hypothalamus, lateral hypothalamic area (LHA), preoptic area, bed nucleus of the stria terminalis, amygdala, and the frontal cortex, regions commonly belonging to the visceral-limbic forebrain (12, 13). From these anatomical data, it does not appear that the predominant effect of the NTS is via the reticular activating system but instead is via limbic forebrain structures, which are implicated in the sleep regulation. There are two ascending pathways, NTS-VM projection and NTS-PBN-PVN/LHA projection. NTS and VM of the thalamus are well known participating in the regulation of sleep-wake homeostasis (19). The low-frequency electrical stimulation of the medullary reticular formation, particularly the dorsal reticular formation and the caudal NTS, produces cortical synchronization indicative of slow wave sleep (SWS) in an awake animal (14). Conversely, lesions of the dorsal reticular formation and of the NTS produced desynchronization of the EEG in a sleeping animal (15). These results suggest the

existence of neurons in the NTS that are involved in regulating sleep. Furthermore, microinjection of morphine into the NTS provokes an enhancement of SWS and this effect is blocked by naloxone (16), suggesting the somnogenic effect of opioidergic system in the NTS. Our previous studies demonstrate that activation of cholinergic system in the caudal NTS of the medulla oblongata mediates the enhancement of non-rapid eye movement (NREM) sleep induced by 10 Hz EA stimulation of Anmian (EX17) acupoints (17). Nonetheless, 10 Hz EA may also increase β-endorphin concentrations in the NTS, which subsequently alters sleep, based upon the evidence of that the NTS area is one of the anatomically distinct β-endorphin pathways in the brain influenced by EA (18). Besides, we also demonstrated the involvement of the NTS opioidergic system in EA-induced alterations in sleep (30). However, the involvement of NTS-VM projections in mediating 10 Hz EA-induced sleep alteration remains unknown. VM receives opioidergic inputs from the NTS and the activation of opioid receptors hyperpolarizes neurons of VM (20). According to the projection pathway, we previously hypothesized that 10 Hz EA alters synaptic strength of the NTS by enhancement and changes the synaptic activity of the VM by downscaling, which contribute to the EA-induced SWS enhancement. This hypothesis has been partially proven by that the EA-induced SWS enhancement was blocked by muscarinic receptor antagonist in the NTS (18). We herein tried to further elucidate whether the changes of

synaptic strength in both NTS and VM are caused by morphological alterations of synaptic density and synaptic size.

3-3 Materials and Methods

3-3-1 Animals

Four male Sprague-Dawley rats (250 ~ 300 g; National Laboratory Animal Breeding and Research Center, Taiwan) were used in this study. The rats were housed separately in individual cages in the isolated room, in which the temperature was maintained at 23 ± 1 ℃ and the light:dark rhythm was controlled in a 12:12 h cycle (40 Watt × 4 tubes illumination). Food (5001 rodent diet, LabDiet) and water were available ad libitum. All procedures performed in this study were approved by the National Taiwan University Animal Care and Use Committee.

3-3-2 Experimental protocol

Two groups of rats were used in the study as follows. Group 1 (n = 4) was used to determine the alteration of synaptic density and synaptic size in the NTS and VM after receiving sham EA stimulation, in which EA stimuli were delivered to the non-acupoints where locate at the ventral conjunction between the forelimb and trunk. Group 2 (n = 4) was used to determine the alteration of synaptic density and synaptic size in the NTS and

VM after receiving 10 Hz EA stimulation at the Amnian acupoints. When 10 Hz EA was given (see later), all rats were lightly anesthetized with ketamine/xylazine, after which rat woke up in 20 to 25 minutes. A twenty-min period of EA stimulation was administered before the onset of the dark period, which is consistent with the protocol described in previous publications (17, 30). The anesthetization was given 25 minutes prior to the dark period onset and lasted for 20 minutes. Both sham and 10 Hz EA stimuli were performed before the dark onset on two consecutive days. The 10 Hz EA stimulus was delivered via the bilateral insertion of stainless needles (32 gauge × 1", Shanghai Yanglong Medical Articles Co.) on Anmian (EX17) points in the depth of 2 mm. The stimulus consisted of a train of biphasic pulses (150 μs duration each) of 10 Hz with intensity of 3 mA, and was delivered by Functions Electrical Stimulator (Trio 300, I.T.O., Japan). The acupoint "Anmian (EX17)" is located at midpoint between Yifeng (TH 17) and Fengchi (GB 20); Yifeng (TH 17) locates posterior to the lobule of the ear in the depression between the mandible and mastoid process; and Fengchi (GB 20) locates in the depression between the upper portion of m. sternocleidomastoideus and m. trapezius in human. The location of Anmian (EX17) in rats is at the relative anatomical location between the strenocleidomastoideus muscle and the splenius capitis muscle, as in the human acupoint map. Sham EA was performed by stimulation of a non-acupoint located at the ventral conjunction between the forelimb and the trunk as previous described (17, 30). Rats were

sacrificed two hours after the second-day sham EA or 10 Hz EA stimuli. Both tissue blocks dissected from NTS and VM were collected. Four slices obtained from the rostral, middle and caudal of NTS and VM were used for observation and counting for the synaptic density and size. Both synaptic density and synaptic size in the NTS and VM were determined by transmission electron microscopy (TEM).

3-3-3 Transmission electron microscopy (TEM)

Brain tissues including NTS and VM were collected and cut into a 0.5 ~ 1.0 mm3 block. The tissue block was fixed in 2.5 % glutaraldehyde in 0.1 M PBS for one hour at 4°C and postfixed in 1 % osmium tetroxide in the same buffer for one hour. The samples were dehydrated in a grade ethyl alcohol series (35 ~ 100 %) then embedded in Spurr's low viscosity resin. Ultrathin sections were made by an UltraCut E microtome (Rankin Biomedical, Holly, MI, USA) and stained with uranyl acetate and lead citrate. Sections were examined with a JEOL JEM-1400 transmission electron microscope at 80 kV.

3-3-4 Statistical analyses for experiment protocol

All values of the synaptic density and synaptic size were presented as the mean ± SEM for the indicated sample sizes. One-way analyses of variance (ANOVA) were performed, comparing between the sham EA group and the 10 Hz EA group. An α level of $p \leqq 0.05$

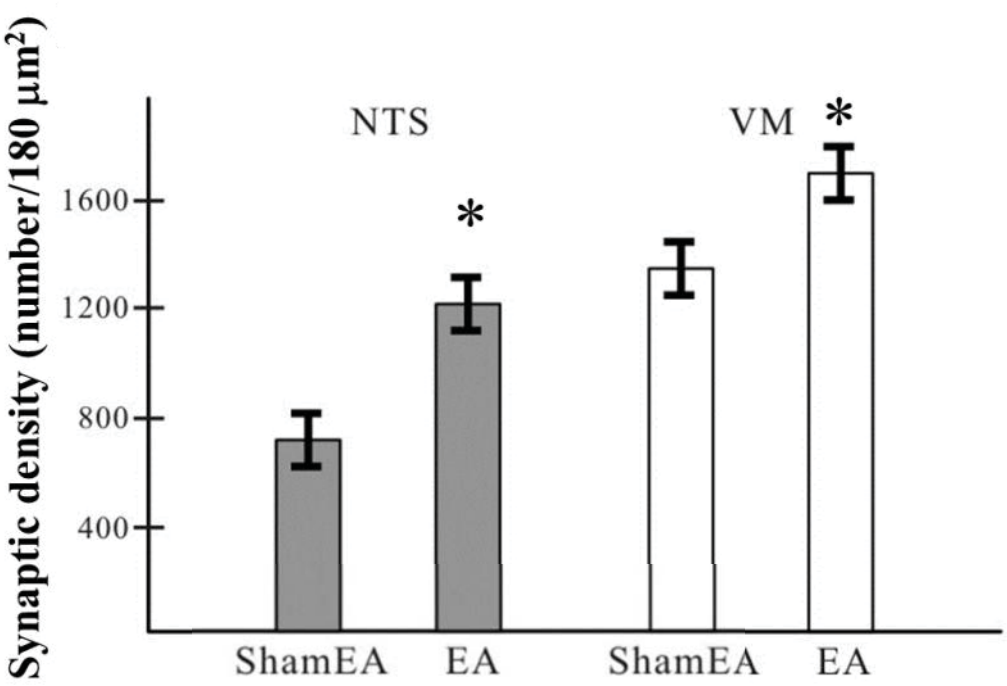
was taken as indicating a statistically significant difference.

3-4 Results

Our results demonstrated that the synaptic density in the NTS was increased after receiving 10 Hz EA stimuli as compared with the results obtained after sham EA stimuli ($p < 0.05$, Figure 6A). Arrow heads in Figure 7 demonstrated the location of synapses in the NTS. The number of synapses was dramatically increased in the NTS after rats receiving 10 Hz EA stimuli (Figure 7). The synaptic density in the VM was also significantly increased after 10 Hz EA stimuli as compared with the results obtained after sham EA stimuli ($p < 0.05$, Figure 6A). Arrow heads in Figure 8 demonstrated the location of synapses in the VM. The number of synapses was also increased in the VM after rats receiving 10 Hz EA stimuli (Figure 8). The total length of synapses counted from the NTS was also enhanced after 10 Hz EA stimulation ($p < 0.05$, Figure 6B). However, the synaptic length counted from the VM was not altered by 10 Hz EA of Anmian acupoints when comparing with the counts obtained after sham EA stimuli (Figure 6B).

Figure 6. The alterations of synaptic density and the total length of synapses in NTS and VM obtained after sham EA stimuli and 10 Hz EA stimulation of Anmian acupoints.

A


Synaptic density (number/180 μm²)
1600
1200
800
400
NTS
VM
*
*
ShamEA
EA
ShamEA
EA

B

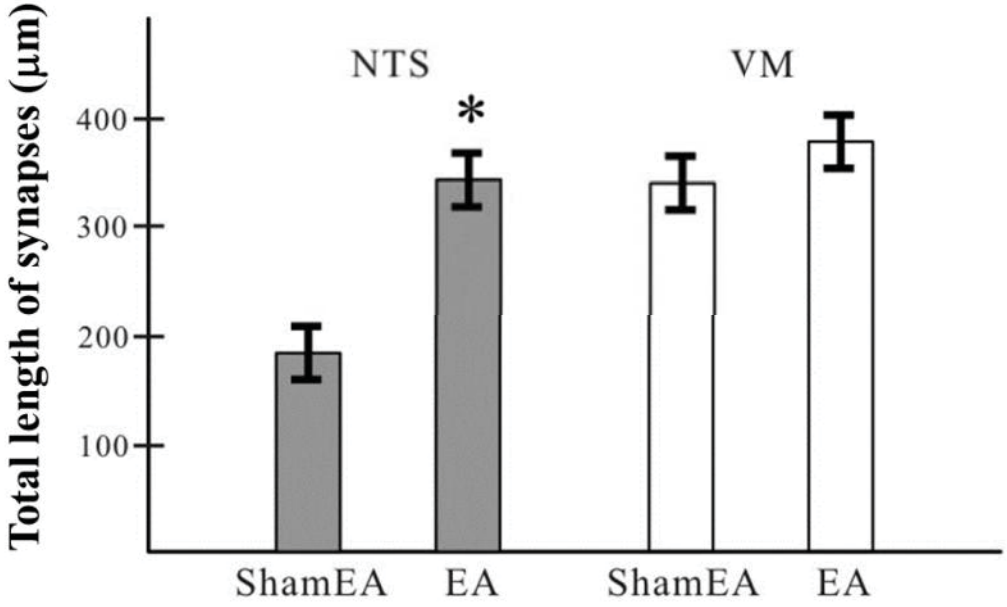
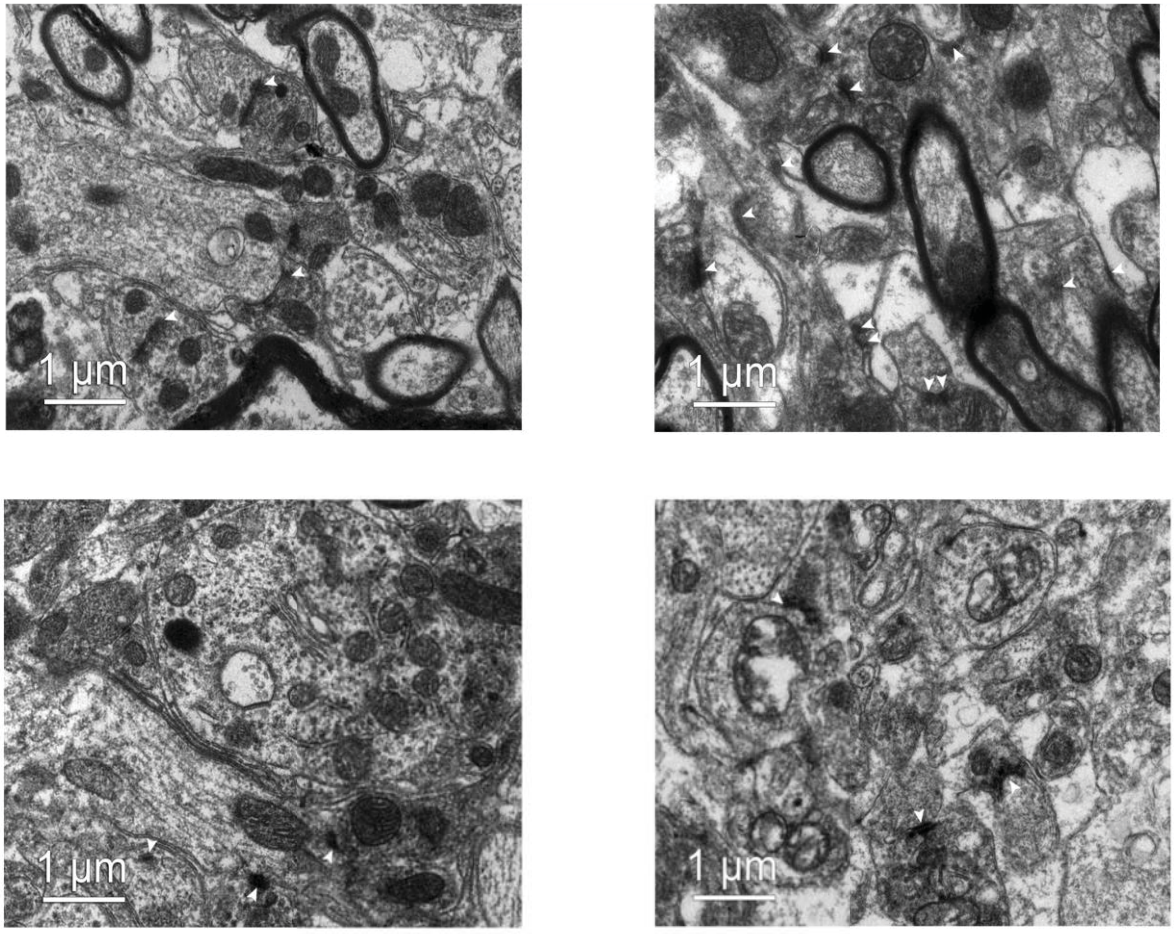

Total length of synapses (μm)
400
300
200
100
NTS
VM
*
ShamEA
EA
ShamEA
EA

Figure 7. The synaptic morphology in the NTS after receiving 10 Hz EA stimuli of Anmian acupints.

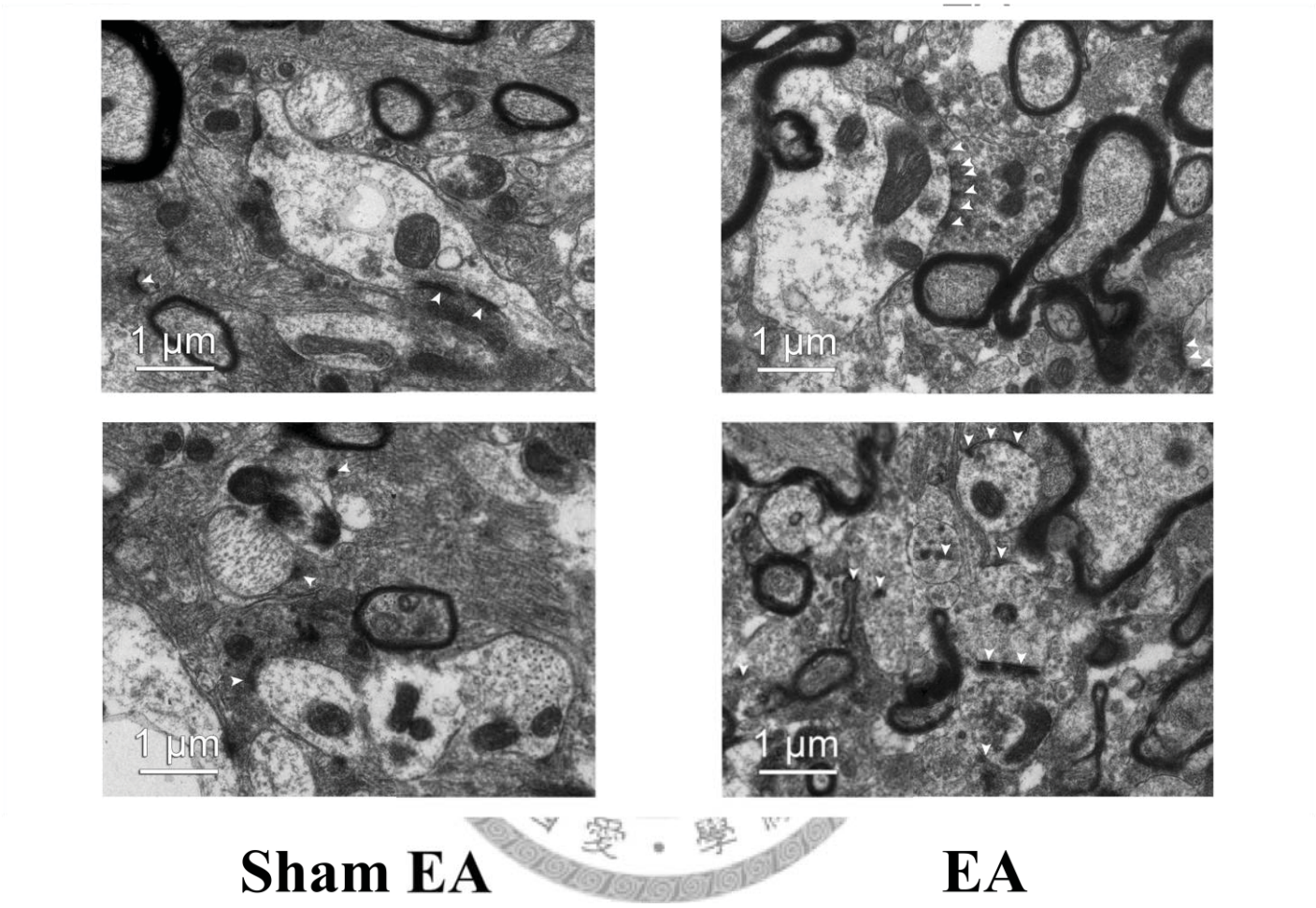
NTS



Sham EA **EA**

Figure 8. The synaptic morphology in the VM after receiving 10 Hz EA stimuli of Anmian acupints.

VM



3-5 Discussion

Our previous studies demonstrated that activation of cholinergic system in the caudal NTS of the medulla oblongata mediates the enhancement of SWS induced by EA stimulation of Anmian (EX17) acupoints (17). Nonetheless, EA may also increase β-endorphin concentrations in the NTS, which subsequently alter sleep, because the NTS area is one of the anatomically distinct β-endorphin pathways in the brain influenced by EA (18). Besides, results of our previous studies also elucidated the involvement of the NTS opioidergic system in EA-induced alterations in sleep (30). The EA-induced enhancement in SWS was dose-dependently blocked by microinjection of the opioid receptor antagonist naloxone and the μ-opioid receptor antagonist naloxonazine into the NTS; administrations of δ-receptor antagonist, natrindole, and the κ-receptor antagonist, *nor*-binaltrophimine, however, did not affect EA-induced alterations in sleep. Furthermore, β-endorphin was significantly increased in both the brainstem and hippocampus after the EA stimuli, which can be blocked by administration of the muscarinic antagonist scopolamine into the NTS. Our findings suggests that mechanisms of EA-induced NREM sleep enhancement may be mediated, in part, by cholinergic activation, stimulation of the opiodergic neurons to increase the concentrations of β-endorphin, and the involvement of the μ-opioid receptors (30).

Although previous results demonstrate the involvement of NTS in the EA of

Anmian-induced sleep regulation, the underlying ascending neural pathways need to be further determined. As aforementioned in the introduction, ascending projections from the NTS are traced through the lateral and dorsal tegmentum and periventricular gray up to the rostral pons and midbrain, PBN, VM of thalamus, PVN of hypothalamus, LHA, preoptic area, bed nucleus of the stria terminalis, amygdala, and the frontal cortex, regions commonly belonging to the visceral-limbic forebrain (12, 13). In addition, it has been indicated that NTS directly projects to thalamus (47), and hypothalamus, including PVN and LHA (48). In this study, the NTS-VM pathway was focused. Anatomic studies revealed that the NTS projects opioidergic efferents directly to the PBN and VM (47), in addition to the glutaminergic excitatory pathways (49,50,51,52). The PBN locates around the superior cerebellar peduncle in the dorsolateral pons (53). The PBN is considered a relay for afferent inputs coming from the NTS to the thalamus and hypothalamus (50), in addition to the direct projections from NTS to VM. The PBN involves in a variety of behavioral and neuroendocrine function, e.g. taste, cardiovascular control, sleep, and threat behavior (54,55,56,57). Granata and Kitai demonstrated that PBN receives afferent innervations from the NTS which elicits an excitatory effect on PBN neurons (50). However, one of recent studies indicates that endomorphinergic neurons, which contain endomorphin-1 and endomorphin-2 that are the selective endogenous ligands for μ-opioid receptors, exist in the NTS and it project efferent to the PBN (20). Other study

also revealed that the distributions of cell soma containing endomorphin-2-immunoreactivity (ir) are prominent in the NTS, and the endomorphin-2-ir varicose fibers locate in both NTS and PBN (58). In addition, endomorphin-2-ir is widely distributed throughout the CNS and associated with brain regions expressing μ-opioid receptors, such as thalamic nuclei and hypothalamic nuclei (58). It also has been shown that the μ-receptor is highly expressed and distributed in the PBN and most thalamic nuclei (e.g. VM), and the distribution of κ-receptor is moderately in the PBN. The synaptic homeostasis hypothesis claims that sleep play a role in the regulation of synaptic strength in the CNS, and it hypothesizes that wakefulness is associated with synaptic potentiation and slow wave sleep is related to the synaptic downscaling (59). It has been declared that the long-term potentiation of synaptic strength occurs when organisms are awake to interact with the environment and to acquire information, which causes EEG activation and the information storage. When sleep occurs, virtual disconnection from the environment triggers depolarizing-hyperpolarizing slow oscillations of the membrane potential which result in highly synchronized EEG. However, the repeated depolarizing-hyperpolarizing sequences cause the down-scaling of the synapses impinging on the postsynaptic neurons (59). Therefore, we hypothesized that EA-induced enhancement of SWS is associated with the changes of synaptic strength in the NTS and VM.

Our results demonstrated that 10 Hz EA of Anmian acupoints significantly enhanced the synaptic density and the total length of synapses in the NTS, suggesting the synaptic plasticity of the cholinergic excitatory inputs has been initiated by morphological changes in synapse and subsequently enhanced synaptic strength in the NTS. Furthermore, the density of synapses in the VM was also increased after 10 Hz EA stimuli, demonstrating the alteration of synaptic plasticity of opioidergic synapses occurred in VM and subsequently downscaled synaptic strength in the VM. However, there was no significant alteration of synaptic length in the VM after receiving 10 Hz EA of Anmian acupoints. These results implicated that increase of synaptic density and synaptic size may partially contribute to the up-regulation of synaptic strength of the NTS after rats received 10 Hz EA stimuli, while the downscaling of VN synaptic strength was only caused by increase of synaptic density.

3-6 Conclusion

In summary, our current results demonstrated that 10 Hz EA stimuli of Anmian (EX17) acupoints changed synaptic morphology in both NTS and VM. The up-regulation of synaptic strength of NTS may explain the mechanism of 10 Hz EA-induced SWS enhancement which we reported previously. Nevertheless, the cause of downscaling of the opioidergic (inhibitory) synaptic strength in the VM needs further investigation by employing electrophysiological recordings.

Chapter 4. Kappa-opioid Receptors in the Caudal Nucleus Tractus Solitarius (NTS) Mediate 100 Hz Electroacupuncture-induced Sleep Activities in Rats

4-1 Abstract

Previous results demonstrated that 10 Hz electroacupuncture (EA) of Anmian acupoints in rats during the dark period enhances slow wave sleep (SWS), which involves the induction of cholinergic activity in the caudal nucleus tractus solitarius (NTS) and subsequent activation of opioidergic neurons and μ-receptors. Studies have shown that different kinds of endogenous opiate peptides and receptors may mediate the consequences of EA with different frequencies. Herein we further elucidated that high frequency (100 Hz) EA of Anmian enhanced SWS during the dark period, but exhibited no direct effect on rapid eye movement (REM) sleep. High frequency EA-induced SWS enhancement was dose-dependently blocked by microinjection of naloxone or κ-receptor antagonist (nor-binaltrophimine) into the caudal NTS, but was affected neither by μ-(naloxonazine) nor δ-receptor antagonists (natrindole), suggesting the role of NTS κ-receptors in the high frequency EA-induced SWS enhancement. Current and previous results depict the opioid mechanisms of EA-induced sleep.

4-2 Introduction

Neuropeptides, along with neurotransmitters, mediate various underlying mechanisms

of neural functions and behaviors (e.g. opioid peptides in pain control (21), corticotrophin-releasing hormone (CRH) in stress-related behavior and sleep-wake regulation (22), hypocretin in feeding behavior and in the maintenance of vigilance states (23), and etc.). Discovery of endogenous opioid peptides, including β-endorphin, dynorphin, enkephalin and endomorphin, in the central nervous system (CNS) reveals the mysterious actions of acupuncture, especially in its analgesic effect. It had first been demonstrated that the acupuncture-induced analgesic effect could be blocked by a broad-spectrum opioid receptor antagonist naloxone in both humans and mice (24,25), implicating the role of endogenous opioid peptides. Chang and Pomeranz had revealed that relatively low doses of naloxone only block the analgesic effect induced by low-frequency (4 Hz) of electroacupuncture (EA) stimulation, but not the consequence induced by high-frequency (200 Hz) of EA (26), suggesting that the low-frequency, rather than the high-frequency, of EA increases the release of endogenous opioids. Nevertheless, Han and his colleagues have further shown that the increase of endogenous opioids mediates the analgesic effects induced by both the low-frequency and high-frequency EA stimuli by employing distinct opioid receptor subtype-specific antagonists (27,28). While μ- and δ-opioid receptors in the spinal cord are dominant in the low-frequency EA-induced analgesia, κ-opioid receptors contribute to the high-frequency EA effects (27, 28). Radioimmunoassay of spinal perfusates from rats

receiving various frequencies of EA stimulations further indicates that 2 Hz EA enhances enkephalin (a mixed μ- and δ-opioid receptor agonist) immunoreactivity (IR), 4 but not the dynorphin (κ-opioid receptor agonist) IR. In contrast, 100 Hz EA increases dynorphin IR rather than enkephalin IR (29).

Our previous results have shown that 10 Hz EA at Anmian (EX17) acupoints increases slow wave sleep (SWS) in rats, which involves the induction of cholinergic activity in the caudal nucleus tractus solitaries (NTS) (17, 30). The NTS is located in the dorsomedial medulla oblongata. Ascending projections from the NTS are traced through the lateral and dorsal tegmentum and periventricular gray up to the rostral pons and midbrain, and terminate in the parabrachial nucleus, which in turn projects to the thalamus, hypothalamus, preoptic area, bed nucleus of the stria terminalis, amygdala, and the frontal cortex, regions commonly belonging to the visceral-limbic forebrain (12, 13). From these anatomical data, it does not appear that the predominant effect of the NTS is via the reticular activating system but instead is via limbic forebrain structures, which are implicated in the sleep regulation. Furthermore, the low-frequency electrical stimulation of the medullary reticular formation, particularly the dorsal reticular formation and the caudal NTS, produces cortical synchronization indicative of SWS in an awake animal (14). Conversely, lesions of the dorsal reticular formation and of the NTS produced desynchronization of the EEG in a sleeping animal (15). These results all

suggest that the existence of neurons in the NTS is involved in generating sleep. Furthermore, microinjection of morphine into the NTS provokes an enhancement of SWS and this effect is blocked by naloxone (16), suggesting the somnogenic effect of opioidergic system in the NTS. The involvement of opioidergic system in EA's therapeutic indications other than the analgesia (e.g. insomnia) has been less discussed in literature. Our previous study has revealed that 10 Hz (low frequency) EA stimulation of Anmian acupoints increases the concentrations of β-endorphin in the brainstem, which consequently enhances SWS through activation of the μ-opioid receptors, rather than the δ- and κ-opioid receptors, in the caudal NTS (30). However, it has never been determined whether different frequencies of EA stimulations at Anmian acupoints activate distinct opioid receptors in the NTS. This current study was designed to clarify what type(s) of opioid receptor is (are) involved in high-frequency (100 Hz) EA-induced sleep alterations.

4-3 Materials and Methods

4-3-1 Pharmacological agents

Stock solutions of a broad spectrum opioid antagonist (naloxone hydrochloride (Tocris, Bristol, UK)), a μ-receptor antagonist (naloxonazine dihydrochloride (Tocris)), a δ-receptor antagonist (naltrindole hydrochloride (Tocris)) and a κ-receptor antagonist

(nor-binaltorphimine dihydrochloride (Tocris)) were dissolved in pyrogen-free saline (PFS). The stock solutions were stored at 4℃ until use. Naloxone, naloxonazine, naltrindole and nor-binaltorphimine were microinjected at three different doses, 0.1, 1.0 and 10 μg/μl. The total volume used for each microinjection was 1 μl.

4-3-2 Animals

Male Sprague-Dawley rats (250 ~ 300 g; National Laboratory Animal Breeding and Research Center, Taiwan) were used in this study. Rats were anesthetized by intraperitonealelectrodes 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 the isolated room, in which the temperature was maintained at 23 ± 1℃ and the light:dark rhythm was controlled in a 12:12 h cycle (40 Watt x 4 tubes illumination). Food (5001 rodent diet, LabDiet) and water were available *ad libitum*. All procedures performed in this study were approved by the National Taiwan University Animal Care and Use Committee.

4-3-3 Experimental protocol

On the 2nd postsurgical day, the rats were connected to the recording apparatus (see below) via a flexible tether. As such, the rats were allowed relatively unrestricted movement within their own cages. Three groups of rats were used in the study as follows: group 1 (n = 8) was used to determine the effects of opioid receptor antagonist (naloxone) on 100 Hz EA-induced alterations in sleep; group 2 (n = 8) was used to depict the effects of μ-receptor antagonist (naloxonazine) on 100 Hz EA-induced sleep alterations; group 3 (n = 8) was used to elucidate the effects of δ-receptor antagonist (naltrindole) and κ-receptor antagonist (*nor*-binaltorphimine) on 100 Hz EA-induced alterations in sleep. One week after rats had adapted to the 12:12-hour light:dark cycle after surgery, a 24-hour undisturbed baseline recordings were obtained beginning at dark onset on the 1st recording day in rats from all groups. When 100 Hz EA was given (see later), all rats were lightly anesthetized with one-third of the dose of ketamine/xylazine used in the surgery, after which rat woke up in 20 to 25 minutes. A twenty-min period of EA stimulation was administered before the onset of the dark period. The anesthetization was given 25 minutes prior to the dark period onset and lasted for 20 minutes. The rationale for carrying out the experiment in the darkness is that rats are active with a lowest level of sleep during the dark period, and a manipulation, if it possesses ability to increase sleep, would significantly augment sleep

during the dark period. In contrast, it may not be easy to enhance sleep during the light period when sleep activity is at its highest circadian level. Since we expected to find a sleep enhancement after the 100 Hz EA stimuli at the Anmian (EX17), we therefore manipulated the EA stimulation before the onset of the dark period and analyzed the sleep alteration during the subsequent dark period. The rats in group 1 were intraperitoneally (IP) administered PFS and microinjected with PFS into the caudal NTS ($_{ip}$PFS+PFS) at 25 minutes prior to the dark onset on two consecutive days, and recordings obtained for 24-h beginning after the second injection. The effects of anesthesia with the NTS microinjection of PFS ($_{ip}$ketamine+PFS) on sleep were determined after IP injection of ketamine/xylazine and the NTS PFS microinjection on two consecutive days. A 100 Hz sham EA ($_{ip}$ketamine+PFS+sham EA) was delivered to control for the non-specific effect of the electrical stimulation, although our previous study had confirmed that no non-specific effect was observed after the sham EA (17, 30). The 100 Hz EA stimuli under anesthesia ($_{ip}$ketamine+PFS+EA) were also performed before the dark onset on two consecutive days, and sleep-wake behavior after the second 100 Hz EA stimulation was then determined. Subsequently, three different doses (0.1, 1.0 and 10 μg) of naloxone ($_{ip}$ketamine+naloxone+EA) were administered 25 minutes prior to the dark onset on the second day of 100 Hz EA stimulation, and the 24-h sleep pattern determined. At least one day without injections was scheduled

between each manipulation. The 100 Hz EA stimulus was delivered via the bilateral insertion of stainless needles (32 gauge x 1", Shanghai Yanglong Medical Articles Co.) on Anmian (EX17) points in the depth of 2 mm. The stimulus consisted of a train of biphasic pulses (150 μs duration each) of 100 Hz with intensity of 3 mA, and was delivered by Functions Electrical Stimulator (Trio 300, I.T.O., Japan). The acupoint "Anmian (EX17)" is located at midpoint between Yifeng (TH 17) and Fengchi (GB 20); Yifeng (TH 17) locates posterior to the lobule of the ear in the depression between the mandible and mastoid process; and Fengchi (GB 20) locates in the depression between the upper portion of m. sternocleidomastoideus and m. trapezius in human. The location of Anmian (EX17) in rats is at the relative anatomical location between the strenocleidomastoideus muscle and the splenius capitis muscle, as in the human acupoint map. Sham EA was performed by stimulation of a non-acupoint located at the ventral conjunction between the forelimb and the trunk as previous described (17). Rats in group 2 received a similar protocol as those in group 1, except that the substance administered was naloxonazine ($_{ip}$ketamine+naloxonazine+EA). Those rats in group 3 underwent a similar protocol as those in groups 1 and 2, except that the substances administered were naltrindole ($_{ip}$ketamine+naltrindole+EA) and *nor*-binaltorphimine ($_{ip}$ketamine+nor-binaltorphimine+EA). There was a week interval between the administrations of three doses of naltrindole and those of three doses of

nor-binaltorphimine.

4-3-4 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 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.

Postacquisition determination of the vigilance state was done by visual scoring of 12-s epochs using custom software (ICELUS, Mark R. Opp) written in LabView for Windows (National Instruments). The animal's behavior was classified as either SWS, REM sleep or waking based on previously defined criteria (60). 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 REM sleep, 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 REM sleep, but power density values in the delta frequency band are generally greater than those in theta frequency band.

4-3-5 Statistical analyses for experiment protocol

All values acquired from sleep-wake recording 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, REM sleep, WAKE) and for sleep architecture parameters were performed, comparing before and after manipulation within subjects, across a certain of time block. An α level of $p \leqq 0.05$ was taken as indicating a statistically significant difference. If statistically significant differences were detected, a *Scheffe post hoc* comparison was made to determine which hourly intervals during experimental conditions deviated from values obtained from the same animals during control conditions.

4-4 Results

4-4-1 The effect of naloxone on the 100 Hz EA-induced alterations in sleep

Anesthetization of rats for 25 minutes with ketamine/xylazine prior to the dark period suppressed both SWS and REM sleep during the first few hours of the dark period, which is consistent with our previous findings and the possible mechanisms was discussed later (17,30). The percentage of time spent in SWS during the first 2-h period after $_{ip}$ketamine+PFS was decreased from 22.4 ± 3.8 % acquired after $_{ip}$PFS+PFS to 5.2 ± 1.4 % ($p < 0.05$; Figure 9A); however, no significant alteration was detected when SWS during the 12-h of the dark period was analyzed in group 1 ($F = 1.269$, non-significance (n.s.); Figure 9B). REM sleep was significantly suppressed from 5.5 ± 0.6 % obtained after $_{ip}$PFS+PFS to 3.3 ± 0.6 % acquired after $_{ip}$ketamine+PFS during the 12-h of the dark period ($F = 18.823$, $p < 0.01$; Figure 9D), especially during the first 3-h after the administrations (Figure 9C). Application of 100 Hz sham EA did not alter any aspect of sleep parameters (data not shown), which is similar to our previous observation (17,30). Twenty-minute of 100 Hz EA stimuli delivered before the dark period on two consecutive days significantly augmented SWS during the post-manipulation hours 5-8 and hour-11 (Figure 9A). Analysis of 12-h dark period revealed that SWS was enhanced from 14.8 ± 1.6 % after $_{ip}$ketamine+PFS to 21.2 ± 1.6 % after $_{ip}$ketamine+PFS+EA ($F = 9.392$, $p < 0.05$; Figure 9B). The percentage of time

spent in SWS during hours 5-8 increased from 17.5 ± 2.1 % obtained after $_{ip}$ketamine+PFS to 29.3 ± 2.6 % after $_{ip}$ketamine+PFS+EA ($p < 0.05$; Figure 9A). The percentage of SWS during the 11-h was also enhanced from 28.0 ± 7.3 % to 36.9 ± 4.2 % ($p < 0.05$; Figure 9B). However, REM sleep was not significantly altered by 100 Hz EA (Figure 9C & 9D).

Administration of three different doses (0.1, 1.0 and 10.0 μg) of naloxone, a broad spectrum opioid antagonist, into the caudal NTS dose-dependently blocked 100 Hz EA-induced increases of SWS, especially during post-manipulation hours 5-8 (Figure 9A & 9B). Across the entire 12-h dark period recording, SWS was suppressed from 21.2 ± 1.6 % after $_{ip}$ketamine+PFS+EA to 16.7 ± 2.1 % after $_{ip}$ketamine+naloxone (10.0 μg)+EA ($F = 18.457$, $p < 0.05$; Figure 10A & 10B), while no significant alteration was detected in REM sleep after administration of nalaxone into the caudal NTS.

4-4-2 The effect of naloxonazine on the 100 Hz EA-induced alterations in sleep

In group 2, the effects of ketamine (suppression of SWS and REM sleep during the first few hours after administrations) and the enhancement of SWS during hours 5-8 after receiving 100 Hz EA stimuli were reproduced as those discovered in group 1 (Figure 10A & 10C). Administration of μ-opioid receptor antagonist, naloxonazine, into the caudal NTS exhibited no significant effect on the 100 Hz EA-induced SWS

enhancement (Figure 10A & 10B). Naloxonazine also had no effect on REM sleep (Figure 10C & 10D).

4-4-3 The effect of naltrindole on the 100 Hz EA-induced alterations in sleep

In group 3, the effects of ketamine (suppression of SWS and REM sleep during the first few hours after administrations) and the enhancement of SWS during hours 5-8 and hours 11-12 after receiving 100 Hz EA stimuli were also reproduced as those found in groups 1 and 2 (Figure 11A & 11C). Administration of δ-opioid receptor antagonist, naltrindole, into the caudal NTS exhibited no significant effect on the 100 Hz EA-induced SWS enhancement (Figure 11A & 11B). Naltrindole had no further effect on REM sleep (Figure 11C & 11D).

4-4-4 The effect of *nor*-binaltorphimine on the 100 Hz EA-induced alterations in sleep

In group 3, our results indicate that the enhancement of SWS during hours 5-8 and hours 11-12 induced by 100 Hz EA stimuli was dose-dependently blocked by the κ-opioid receptor antagonist, *nor*-binaltorphimine. The percentage of time spent in SWS across the entire 12-h dark period was suppressed from 25.6 ± 2.6 % obtained after $_{ip}$ketamine+PFS+EA to 21.1 ± 2.6 % ($F = 3.574$, n.s.), 19.3 ± 2.4 % ($F = 9.784$, $p < 0.05$) and 19.0 ± 2.4 % ($F = 9.897$, $p < 0.05$; Figure 12A & 12B) after administrations of 0.1,

1.0 and 10 μg of *nor*-binaltorphimine, respectively. The effect of *nor*-binaltorphimine on blocking the 100 Hz EA-induced SWS enhancement was dominantly occurred during hours 4-7 and hours 11-12 (Figure 12A). *Nor*-binaltorphimine exhibited no effect on REM sleep (Figure 12C & 12D).

Figure 9. The effects of high frequency (100 Hz) stimulation by EA at Anmian (EX17) acupoints on vigilance states and the effect of naloxone. Shade area: $_{ip}$PFS+PFS, open circles: $_{ip}$ketamine+PFS, closed circles: $_{ip}$ketamine+PFS+EA, open triangles: $_{ip}$ketamine+naloxone (10 μg)+EA. * represents a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS and $_{ip}$PFS+PFS. § depicts a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS+EA and $_{ip}$ketamine+PFS. # demonstrates a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS+EA and $_{ip}$ketamine+naloxone+EA. The black horizontal bar on the x-axis represent the dark period of the 12:12h light:dark cycle. REM refers to rapid eye movement; SWS, slow wave sleep.

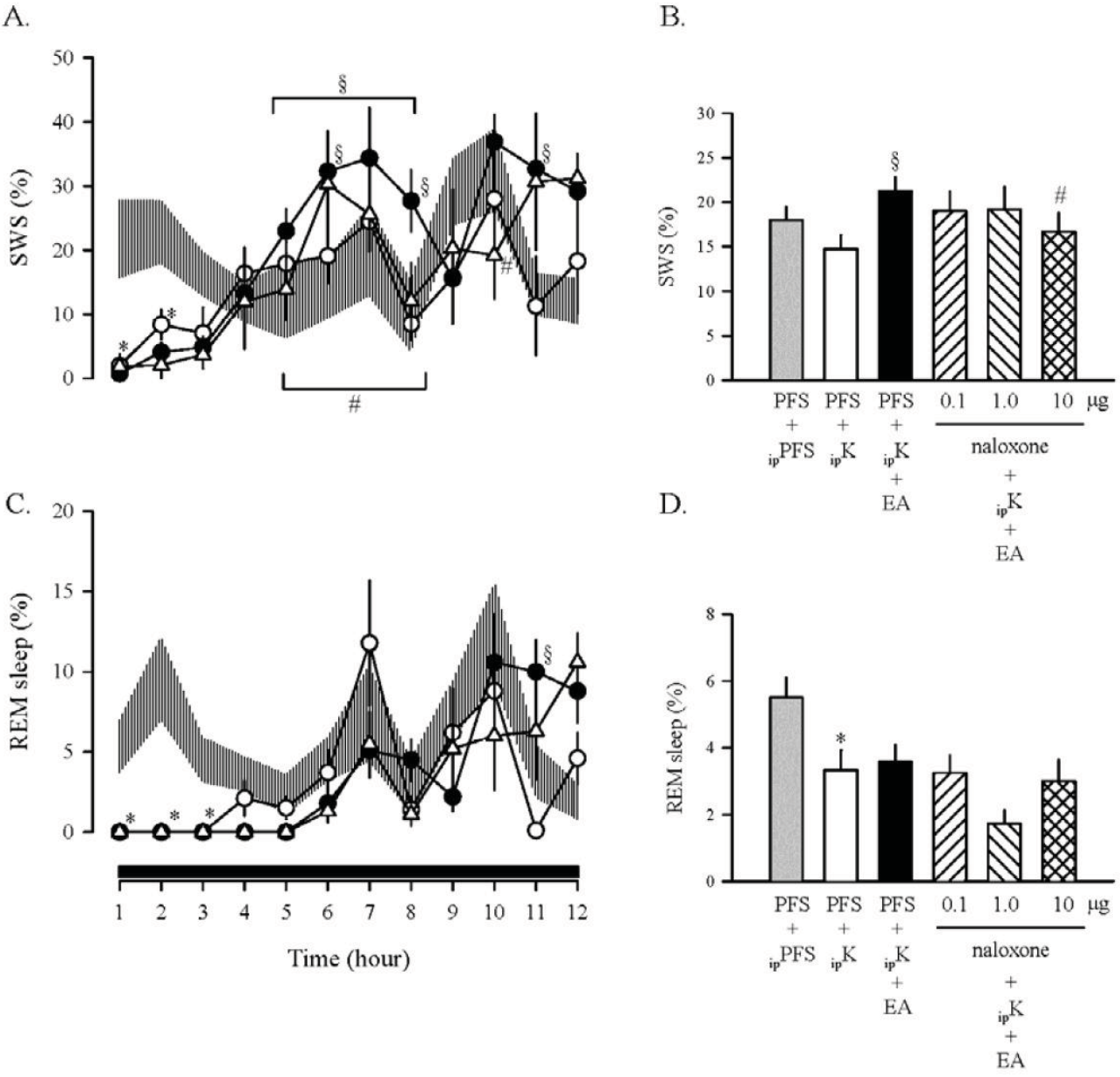

A.
B.
C.
D.
SWS (%)
REM sleep (%)
Time (hour)
PFS + ipPFS
PFS + ipK
PFS + ipK + EA
0.1
1.0
10 µg
naloxone + ipK + EA

Figure 10. The effects of naloxonazine on 100 Hz EA-induced alterations in sleep. Shade area: $_{ip}$PFS+PFS, open circles: $_{ip}$ketamine+PFS, closed circles: $_{ip}$ketamine+PFS+EA, open triangles: $_{ip}$ketamine+naloxonazine (10 μg)+EA. * represents a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS and $_{ip}$PFS+PFS. § depicts a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS+EA and $_{ip}$ketamine+PFS.

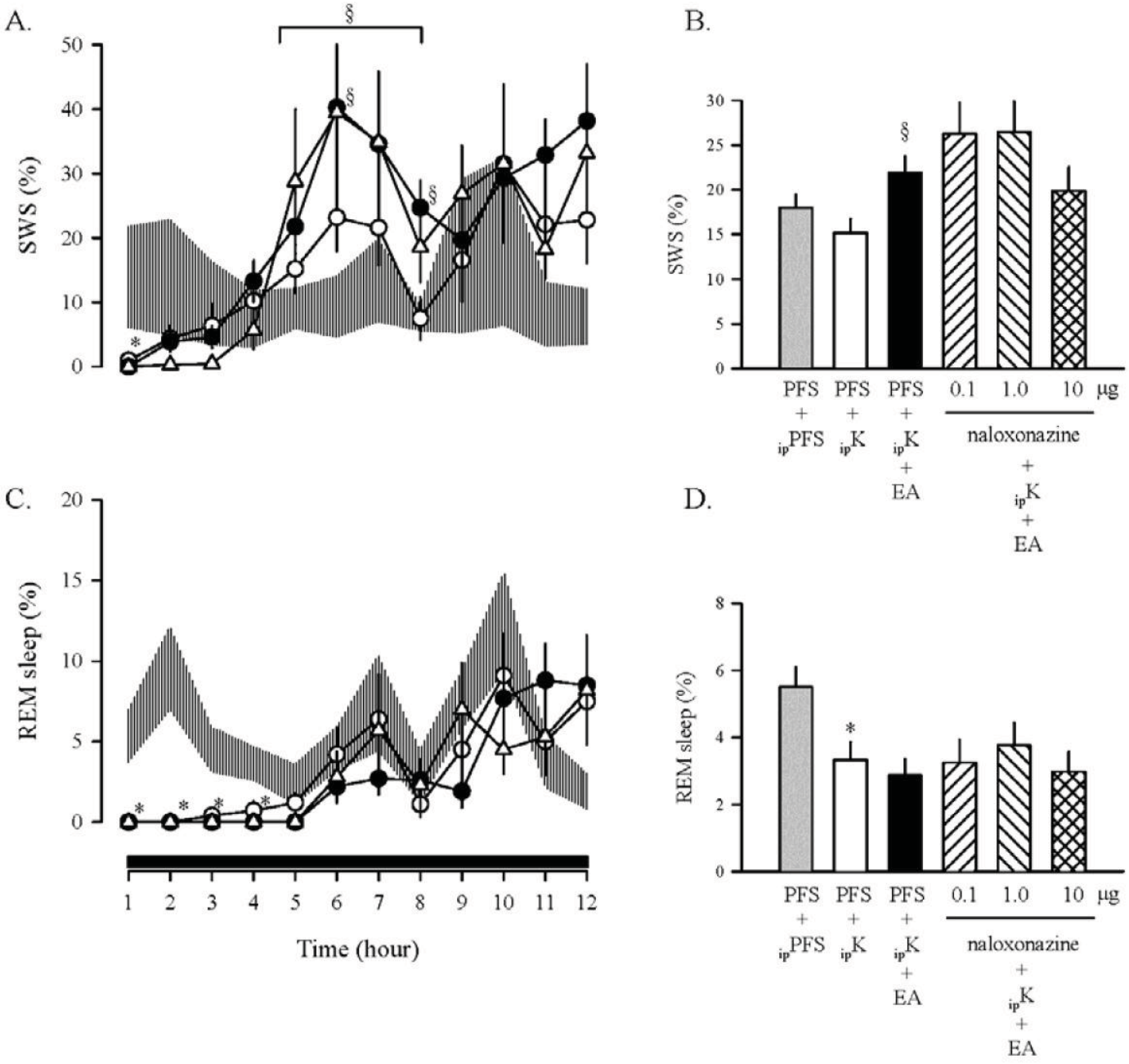

A.
SWS (%)
50
40
30
20
10
0
§
§
§
*
B.
SWS (%)
30
25
20
15
10
5
0
§
PFS + ipPFS
PFS + ipK
PFS + ipK + EA
0.1
1.0
10 µg
naloxonazine + ipK + EA
C.
REM sleep (%)
20
15
10
5
0
*
*
*
*
1 2 3 4 5 6 7 8 9 10 11 12
Time (hour)
D.
REM sleep (%)
8
6
4
2
0
*
PFS + ipPFS
PFS + ipK
PFS + ipK + EA
0.1
1.0
10 µg
naloxonazine + ipK + EA

Figure 11. The effects of naltrindole on 100 Hz EA-induced alterations in sleep. Shade area: $_{ip}$PFS+PFS, open circles: $_{ip}$ketamine+PFS, closed circles: $_{ip}$ketamine+PFS+EA, open triangles: $_{ip}$ketamine+naltrindole (10 μg)+EA. * represents a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS and $_{ip}$PFS+PFS. § depicts a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS+EA and $_{ip}$ketamine+PFS.

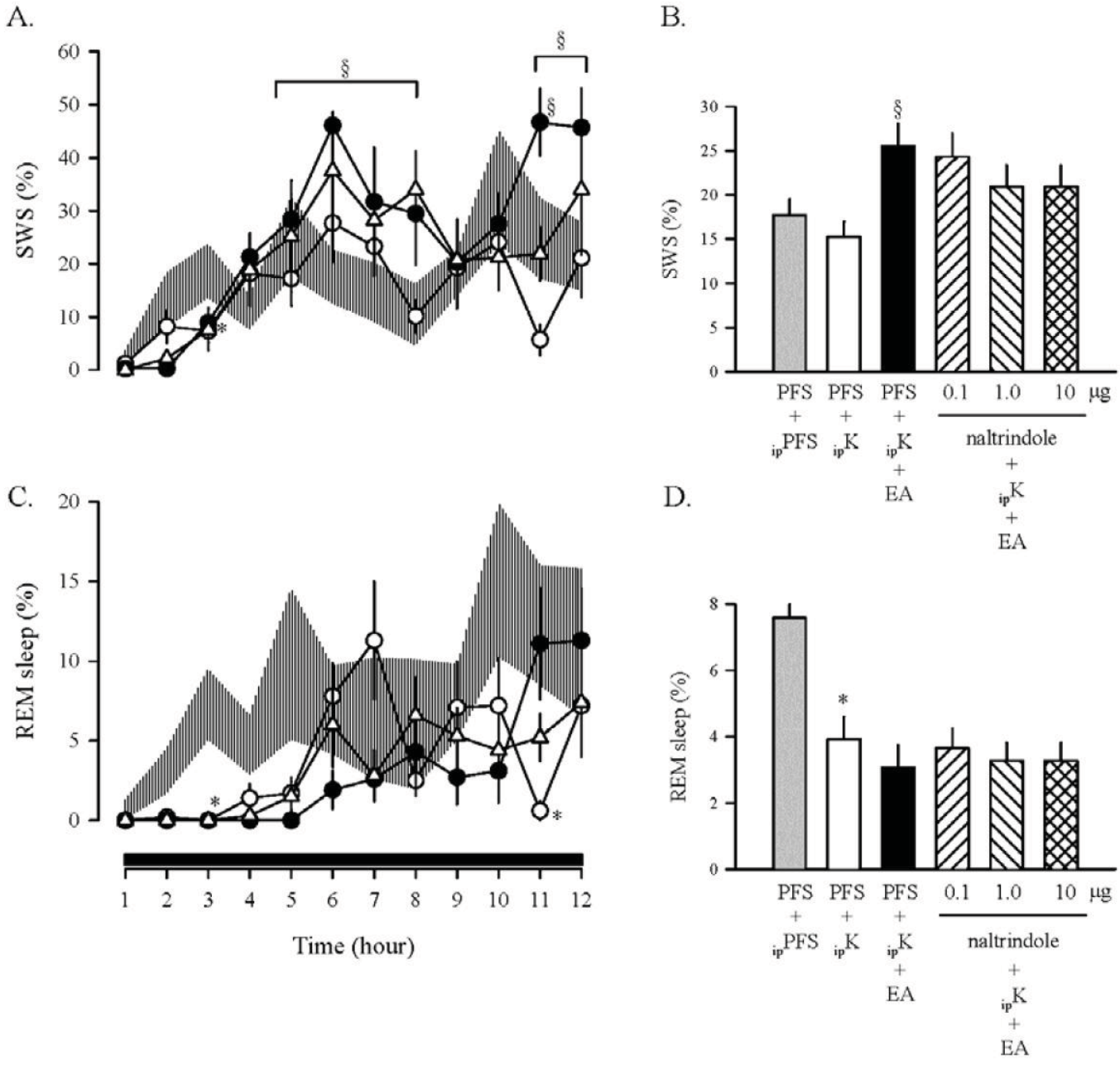

A.
SWS (%)
60
50
40
30
20
10
0
§
§§
*
B.
SWS (%)
30
25
20
15
10
5
0
§§
PFS + ipPFS
PFS + ipK
PFS + ipK + EA
0.1
1.0
10 µg
naltrindole + ipK + EA
C.
REM sleep (%)
20
15
10
5
0
*
*
1 2 3 4 5 6 7 8 9 10 11 12
Time (hour)
D.
REM sleep (%)
8
6
4
2
0
*
PFS + ipPFS
PFS + ipK
PFS + ipK + EA
0.1
1.0
10 µg
naltrindole + ipK + EA

Figure 12. The effects of nor-binaltorphimine on 100 Hz EA-induced alterations in sleep. Shade area: $_{ip}$PFS+PFS, open circles: $_{ip}$ketamine+PFS, closed circles: $_{ip}$ketamine+PFS+EA, open triangles: $_{ip}$ketamine+*nor*-binaltorphimine (10 μg)+EA. * represents a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS and $_{ip}$PFS+PFS. § depicts a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS+EA and $_{ip}$ketamine+PFS. # demonstrates a statistically significant difference between the values obtained from $_{ip}$ketamine+PFS+EA and $_{ip}$ketamine+*nor*-binaltorphimine+EA.

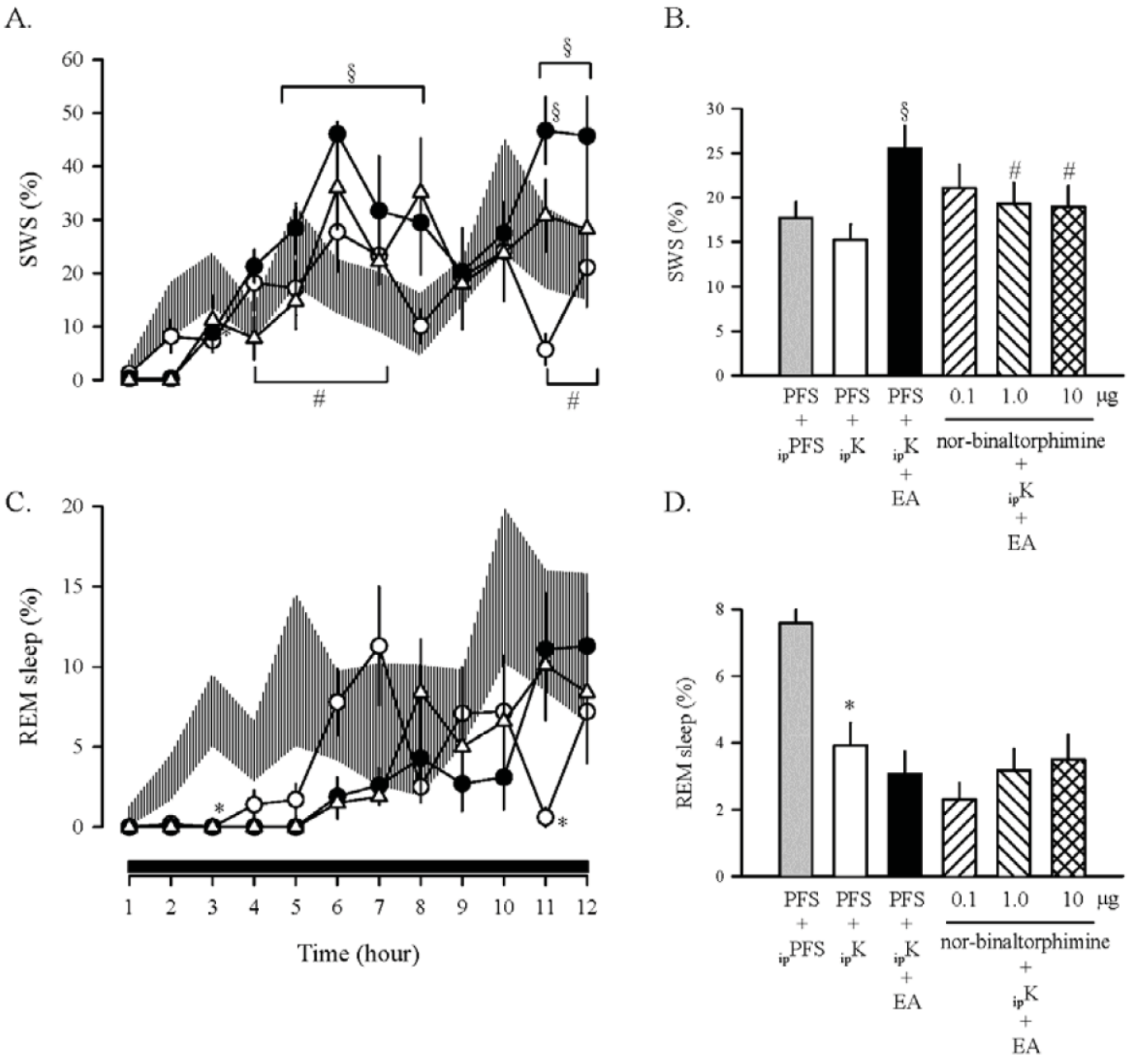
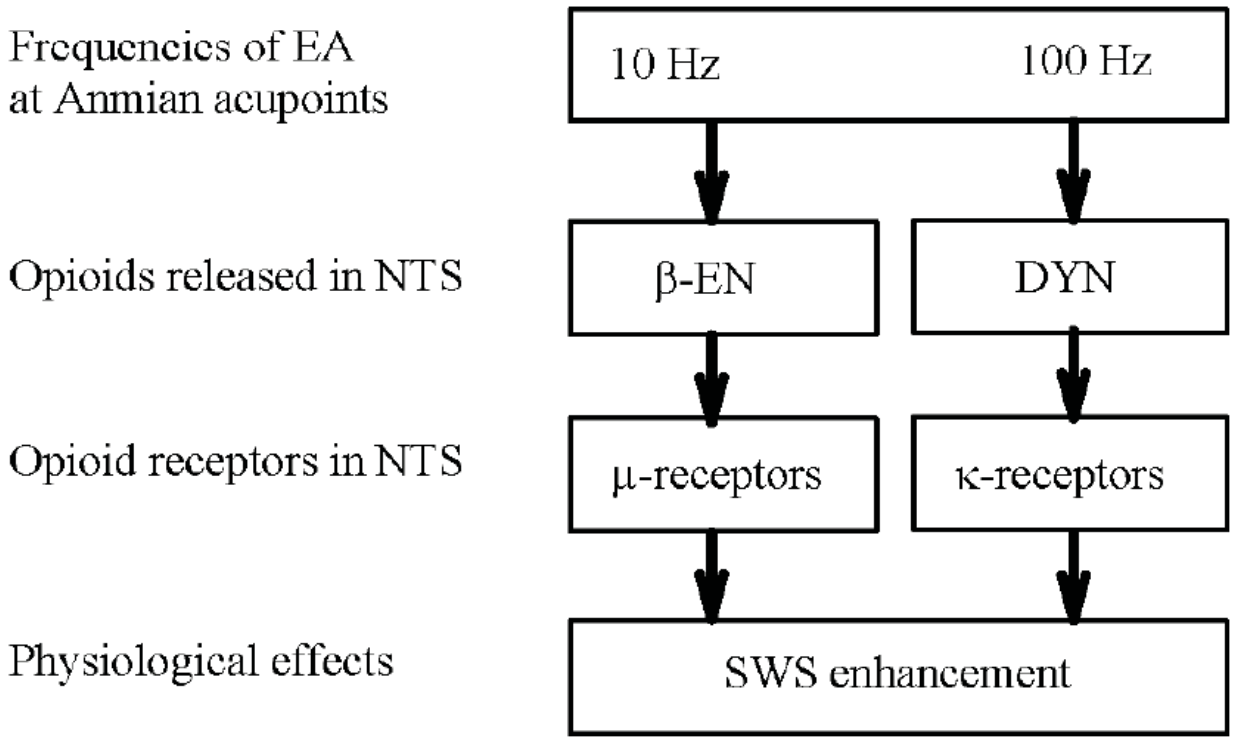

A.
SWS (%)
60
50
40
30
20
10
0
§
§
§
#
#
B.
SWS (%)
30
25
20
15
10
5
0
§
#
#
PFS + ipPFS
PFS + ipK
PFS + ipK + EA
0.1
1.0
10 μg
nor-binaltorphimine + ipK + EA
C.
REM sleep (%)
20
15
10
5
0
*
*
1 2 3 4 5 6 7 8 9 10 11 12
Time (hour)
D.
REM sleep (%)
8
6
4
2
0
*
PFS + ipPFS
PFS + ipK
PFS + ipK + EA
0.1
1.0
10 μg
nor-binaltorphimine + ipK + EA

Figure 13. A hypothetical model by which different frequencies of EA Anmian (EX17) alter SWS through different opioid receptors in the caudal NTS. β-EN: β-endorphin; DYN: dynorphin.


Frequencies of EA
at Anmian acupoints
10 Hz
100 Hz
Opioids released in NTS
β-EN
DYN
Opioid receptors in NTS
μ-receptors
κ-receptors
Physiological effects
SWS enhancement

4-5 Discussion

Acupuncture and electroacupuncture (EA) have been recommended as an alternative medicine for several therapeutic indications by the World Health Organization (WHO), such as alleviation of pain, reduction of inflammation and management of insomnia. The theory underlying EA is still controversial, although the action of EA has been widely discussed in literature. The discovery of endogenous opioid peptides, including enkephalin, β-endorphin, dynorphin and endormorphin, since 1970's enhances the investigation of underlying mechanisms of EA, especially in the EA-induced analgesia. Three main receptor subtypes of the opioid receptors, including the μ-, δ- and κ-opioid receptors, in the spinal cord have been indicated involving in the mechanisms of

EA-induced analgesia. Endormorphin and dynorphin are respectively considered as the relatively pure μ- and κ-opioid receptor agonists (61,62), while enkephalin and β-endorphin are mixed μ- and δ- opioid receptor agonists (review (21, 63). Han and his colleagues have revealed that low frequency (2 Hz) EA increases met-enkephalin, but not dynorphin, in the spinal cord; while high frequency (100 HZ) EA increases the release of dynorphin rather than that of met-enkephalin (29). The stimulation of EA between low and high frequency (e.g. 15 Hz) activates both enkephalins and dynorphins (29). They further demonstrated that the analgesic effect induced by low-frequency EA stimulation is mediated by μ- and/or δ-opioid receptors; in contrast, high-frequency

EA-induced analgesia is mediated by κ-opioid receptors (27, 28). These observations suggest that different endogenous opioid peptides would be released and act on distinct opioid receptors in the spinal cord under different stimulating conditions of EA. However, the involvement of opioid receptors in sleep alteration under different stimulating conditions of EA remains undetermined.

We previously demonstrated that 10 Hz EA stimuli of Anmian (EX17) acupoints in anesthetized rats for 20 minutes prior to the beginning of the dark period of the light:dark cycle in two consecutive days enhances SWS during the subsequent dark period (17,30). Our results also implicated that 10 Hz EA-induced SWS enhancement may be mediated, in part, by vagal cholinergic afferents to the caudal NTS (17, 30). There are two anatomically distinct β-endorphin pathways in the brain; the major pathway originates in the arcuate nucleus and the minor one is in the area of the NTS of the caudal medulla (18). Furthermore, dynorphin is also expressed in neurons of the brainstem NTS (64). The anatomical distributions of opioid receptors and peptides in the CNS have well described by Mansour et al. in 1988 (65). The neurons containing pro-opiomelanocortin (POMC), pro-enkephalin and pro-dynorphin are abundant in the NTS (65). The μ- and κ-opioid receptors are localized in the caudal NTS with a highly density, however little or no δ-opioid receptor can be observed in the brainstem, including the NTS (65). Our previous results have depicted that the SWS enhancement

after 10 Hz EA of Anmian acupoints is mediated by increasing the concentrations of β-endorphin and activation of the μ-opioid receptors in the NTS (30). Herein we tried to further elucidate whether high frequency (100 Hz) EA stimulation of Anmian acupoints also elicits similar effect of sleep enhancement and whether the sleep alterations under different EA stimulation conditions are mediated by distinct opioid receptors. Our current results demonstrated that 100 Hz (high frequency) EA stimuli of Anmian (EX17) acupoints in anesthetized rats for 20 minutes in two consecutive days enhanced SWS, but not REM sleep, during the subsequent dark (active) period. The enhancement of SWS after 100 Hz EA is similar to that after 10 Hz EA stimulation (30), indicating both low-frequency (10 Hz) and high-frequency (100 Hz) EA stimuli possesses ability to alter SWS in the same direction. Application of naloxone, a broad spectrum of opioid receptor antagonist, was used to determine the involvement of NTS opioidergic receptors in the 100 Hz EA-induced increase of SWS. We found that administration of naloxone directly into the caudal NTS dose-dependently blocked 100 Hz EA-induced SWS enhancement during the dark period, implicating the involvement of endogenous opiates in the caudal NTS. A potential role for three major opioid receptors, μ-, δ- and κ-receptors, was then determined by application of specific receptor antagonists. Our results demonstrated that *nor*-binaltorphimine, a κ-opioid receptor antagonist, exhibited a similar dose-dependent effect on blocking 100 Hz EA-induced SWS enhancement as

that of naloxone; whereas naloxonazine (a μ-opioid receptor antagonist) and naltrindole (a δ-opioid receptor antagonist) had no effect. These observation combining with our previous results — μ-opioid receptors mediate 10 Hz EA-induced SWS enhancement (30) — suggest that distinct opioid receptors in the NTS involve in different stimulation frequencies of EA-induced SWS enhancement, which is similar to the underlying mechanisms of EA-induced analgesia in the spinal cord as reported by Han and his colleagues (21,27).

In order to perform the 100 Hz EA stimulation easily, rats were lightly anesthetized. We found that both SWS and REM sleep during the first few hours of the dark period were decreased after rats recovered from the ketamine anesthetic. The decreases in SWS and REM sleep were also observed in our previous study (17,30). Ketamine, a cyclohexanone derivative, is used clinically as a dissociative anesthetic agent both in humans and animals. Ketamine is a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist that blocks cation channels (37). It has been demonstrated that administration of ketamine or MK-801, another NMDA receptor antagonist, at sub-anesthetic doses produces a robust, dose-dependent increase in the intensity of δ-power of the NREM sleep (38,39). Furthermore, the effect of MK-801 by increasing the metabolic rate in the hippocampus and other limbic structures stimulates physiological sleep that is similar to the sleep that follows sleep deprivation, indicating the need of homeostatic recovery (40).

Therefore, the suppression of NREM and REM sleep after recovery from the ketamine anesthetization during the beginning of the dark period may be due to a homeostatic compensation to the previous anesthetic state. However, this explanation needs to be further investigated.

4-6 Conclusion

In summary, our current results demonstrated that 100 Hz EA stimuli of Anmian (EX17) acupoints enhance SWS and this enhancement is blocked by naloxone and *nor*-binaltorphimine, implicating the mediation of κ-opioid receptors. Comparing to our previous observation of involvement of μ-opioid receptors in 10 Hz EA-induced SWS enhancement, we concluded that μ-opioid receptors in the caudal NTS mediate the low-frequency (10 Hz) EA-induced SWS alteration, while κ-opioid receptors mediate the high-frequency (100 Hz) EA-induced SWS enhancement. A diagram elucidating one hypothetical mechanism by which different frequencies of EA at Anmian (EX17) alters sleep is depicted in Figure 13.

Chapter 5. Conclusions

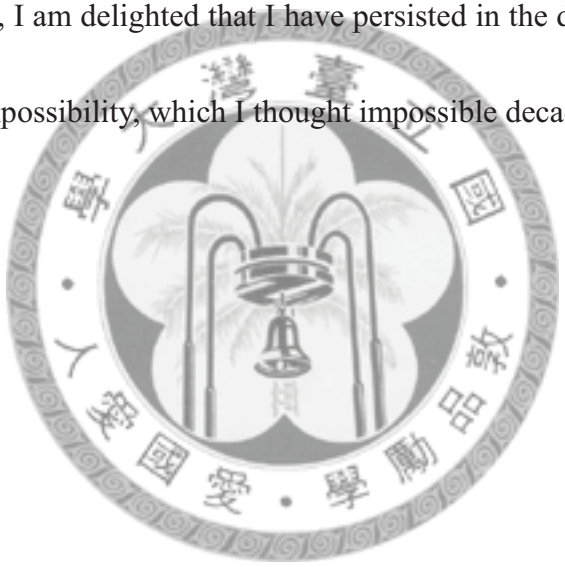
In summary, our current results demonstrate that EA stimuli of Anmian (EX17) acupoints enhance NREM sleep and this enhancement is blocked by naloxone and naloxonazine, while inhibiting μ-opioid receptors from functioning. Furthermore, the activation of NTS muscrainic receptors after low-frequency EA stimuli increases concentrations of β-endorphin, which mediates the enhancement of NREM sleep after EA stimuli of Anmian (EX17) acupoints. Depicted in Fig. 5 is a diagram elucidating one hypothetical mechanism by which EA of Anmian (EX17) alters sleep.

Other results demonstrated that, in conclusion, 100 Hz EA stimuli of Anmian (EX17) acupoints enhance NREM sleep and this enhancement is blocked by naloxone and nor-binaltorphimine, implying the mediation of κ-opioid receptors. By comparing our previous observations of involvement of μ-opioid receptors in the enhancement of 10 Hz EA-induced NREM sleep with new ones, we concluded that μ-opioid receptors in the caudal NTS mediated the low-frequency (10 Hz) EA-induced NREM sleep alteration, while κ-opioid receptors mediated the high-frequency (100 Hz) EA-induced NREM sleep enhancement. Depicted in Figure 5 is a diagram elucidating one hypothetical mechanism by which different frequencies of EA at Anmian (EX17) alters sleep.

All told, our current results demonstrated that 10 Hz EA stimuli of Anmian (EX17) acupoints changed synaptic morphology in both NTS and VM. The up-regulation of

synaptic strength of NTS may explain the mechanism of 10 Hz EA-induced NREM sleep enhancement which we reported previously. Nevertheless, the cause of downscaling of the opioidergic (inhibitory) synaptic strength in the VM calls for further investigation by employing electrophysiological recordings.

After long years of labor, some work has been done, what by electroacupuncture-induced sleep in rats and what by persistence-induced dream in myself. Looking back, I am delighted that I have persisted in the devotion that managed to open the door of impossibility, which I thought impossible decades ago. Thank God!



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Appendix：Publication list (2006~2011)

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