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缺乏睡眠對大白鼠傷害感受及 c-fos 表現上的效應(2/2)

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（計畫名稱）

Effects of Sleep Deprivation on nociception and c-fos Expression in Rats

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中 文 摘 要

本實驗乃採取前瞻性之單盲隨機設計，分別以 52隻 250-350 公克大白鼠來研究缺乏睡眠影響傷害感受的機轉。本報告包含兩個在三年內完成之實驗計劃報告。

第一年；實驗一：Naloxone 對缺乏睡眠提升大白鼠疼痛閾值的效應。

背景：我們的研究曾經發現在大白鼠缺乏睡眠一、三及五天分別明顯延長擺尾潛伏期 (Tail flick latency) 35%、36% 及 30% 之久。倘若缺乏睡眠是經鴉片途徑影響疼痛閾值，則像 Naloxone 一類的嗎啡拮抗劑，將可拮抗缺乏睡眠對疼痛的效應。本實驗之目的在研究 Naloxone 對缺乏睡眠提升大白鼠疼痛閾值的效應

方法：把十六隻大白鼠，個別飼養在一個未啟動的剝奪睡眠飼養器內三天，讓其適應環境後，進行擺尾測試以記錄其基礎值。其後、再隨機將大白鼠分成 A 及 B 兩組。A、 B 組老鼠分別在無法睡眠及有自由睡眠下度過 24 小時。在隔天早上再進行擺尾測試，並記錄測試結果。在完成測試後，給予 naloxone 1mg/kg i.p. 5 分鐘後，再進行並記錄擺尾測試。隨後將老鼠放回籠中，給予充份休息五天後再交替兩組睡眠條件，即 A 組有自由睡眠而 B 組在無法睡眠下讓其再度過另一24 小時，最後再依上述步驟測量各種條件下之疼痛閾值。在完成所有資料之收集後，以 Student's t-test 分析比較自由睡眠與缺乏睡眠前、後及給予 naloxone 後擺尾測試之效應。

結果：我們的研究發現 A 、B 組大白鼠在缺乏睡眠 24 小時後分別較自由睡眠後有明顯地延長擺尾潛伏期 (Tail flick latency) 33.4%±2.6% （P=0.006）及 27.0%±2.7%（P=0.007）之久。所有缺乏睡眠大白鼠在給予 naloxone 1mg/kg i.p. 五分鐘後，其擺尾潛伏期均回復到實驗開始時的基礎值。

第二、三年；實驗二：缺乏睡眠對大白鼠皮下注射福馬林後行為反應及脊髓背角 c-fos 活動表現上的效應。

背景：因擺尾測試只能測量動物瞬間短暫疼痛之反應，故其研究結果仍是未能完整說明缺乏睡眠影響傷害感受的機轉。福馬林測試（formalin test）可量化測量長期疼痛刺激的行為反應，故較為近似真實臨床下的疼痛傷害。本實驗之目的在比較缺乏睡眠及允許自由睡眠大白鼠在皮下注射福馬林後行為反應及脊髓背角 c-fos 活動表現上的差異性。

方法：本計劃把三十六隻大白鼠隨機分成 C1、C2、C3、D1、D2 及 D3 六組。先讓老鼠適應環境三天。再分別讓 D1、D2 及 D3 組大白鼠缺乏睡眠 1、2 及 3 天後給予福馬林測試。C1、C2、C3 組大白鼠則在適應期後另加 1、2 及 3 天自由睡眠才進行測試。在測試後一小時內記錄大白鼠對福馬林疼痛之行為反應。並在犧牲後記錄脊髓背角 c-fos 之活動表現。並在完成資料蒐集後，分析比較各相關資料。

結果：

C1、C2、C3、D1、D2 及 D3 六組之大白鼠對施打福馬林後，在疼痛行為反應上並無明顯的差異。然而脊髓背角 c-fos 之活動表現（Number of c-fos-like immunoreactive neurons）却在缺乏睡眠 2 及 3 天後較對照組有明顯增加現象。此增加現象遍佈於脊髓背角的 Superficial (I-II), Nucleus Proprius (III-IV) 及 Neck (V) lamina 內。

英文摘要

The aim of this project is to assess the effect of total sleep deviation on the reaction of rats subjected to thermal and chemical noxious stimuli.

Materials and Methods: Two different experiments were performed to assess the sensitivity to thermal (tail flick) and chemical (behavior reaction and c-fos immunoreactivity of the spinal cord in rats after subcutaneous formalin injection) noxious stimuli. Total sleep deprivation was elicited using the Dish-on-water method.

First year: Experiment 1: The effects of naloxone on increased pain threshold after total sleep deprivation
The aim of the study is to study the effect of naloxone on increased pain threshold after total sleep deprivation.
Sixteen male SD rats were first placed into a nonfunctioning sleep deprivation apparatus for 3 days of adaptation. Tail-flick test were performed on all rats after adaptation and the rats were then randomly assigned into two groups: group A (n=8) and group B (n=8). Each rat was randomly subjected to either one of the two different conditions for 24 hours: condition I, total sleep deprivation; condition II, adequate rest (the rats were left undisturbed). Group A rats experienced condition I first followed by condition II, whereas, group B rats experienced condition II first and then condition I. The rats were allowed to rest in situ for a period of five days in between the two conditions so that the rats can have enough rest in between study.

Tail-flick test were performed and results recorded on the morning after total sleep deprivation. After this recording, naloxone 1mg/kg i.p. was injected and tail-flick test were performed and recorded 5 minutes later.

Results:

For the thermal experiment, the percentage TFL increased after 24 h of total sleep deprivation compared to value before conditioning was 33.4%±2.6% (p=0.007) in group A rats and 27.0%±2.7% (p=0.007) in group B rats. However, after naloxone injection, tail flick latency returned to the baseline value.

Second & third year: Experiment 2: The effects of total sleep deprivation on behavior response and c-fos expression of formalin test of rats.

For chemical noxious stimuli, 36 rats were randomly allocated into 6 groups, group C1, C2, C3, D1, D2 and D3. Before the study, all rats were placed into a nonfunctioning sleep deprivation apparatus for 3 days of adaptation. Rats from Group D1, D2 and D3 were subjected to 1, 2 and 3 days of total sleep deprivation respectively. Group C1, C2, and C3 rats were subjected to an extra 1, 2 and 3 days of adaptation respectively. Formalin test was applied to Group D1, D2 and D3 rats on the morning of the second day after confinement. Group C1, C2, and C3 rats had the test immediately after their total adaptation period. Immediately after formalin injection, behavior reaction was recorded for an hour. The rats were sacrificed and c-fos immunoreactivity of the spinal cord was examined. Data between groups will be analyzed and compared.

Data were compared and analyzed by Student's t-test to see if the total sleep deprivation or naloxone effects on tail flick responses were significant and whether behavior reaction and c-fos immunoreactivity of the spinal cord in rats after subcutaneous formalin injection were different between groups.

Results:

For the chemical noxious stimuli:

Total sleep deprivation of 1, 2 and 3 days and their corresponding control groups do not have any effects on behavioral response to formalin injection. However, c-fos response (Number of c-fos-like immunoreactive neurons) to formalin injection was significantly greater in 2 and 3 days of total sleep deprivation compared with liberal sleep environment. This increase was observed in the Superficial (I-II), Nucleus Proprius (III-IV) and Neck (V) lamina of the dorsal horn.

Conclusion:

Total sleep deprivation induced a significant decrease in the behavioral response to noxious thermal stimuli in rats which can be reversed with the injection of naloxone. Total sleep deprivation of 1, 2 and 3 days did not have any effects on behavioral response to formalin injection. However, c-fos response (Number of c-fos-like immunoreactive neurons) to formalin injection was significantly greater in 2 and 3 days of total sleep deprivation compared with liberal sleep environment.

關鍵詞

缺乏睡眠；大白鼠；傷害感受；Naloxone；福馬林測試；c-fos 細胞致癌基因。

Sleep deprivation; Rats; Nociception; Naloxone; Formalin test; c-fos.

目　錄

報 告 內 容

前言，文獻探討及研究目的

Background

Pain and sleep disturbance are two universal and challenging issues to clinical physicians. Pain and sleep disturbance affect each other very much, but it is difficult to determine the direction of cause between pain and sleep disturbance. Understanding the co-relationship between sleep disturbances and pain may provide better quality of life to pain patients and help to modify the strategy of pain management especially for pain which poorly responds to conventional analgesics. Currently the majority of studies that suggest a relationship between pain and sleep are supported only by correlational evidence. Morin et al.[1] described that pain may disturb or interrupt sleep. They found that chronic pain patients frequently suffered from sleep disturbances. However, sleep disturbances may also influence pain intensity and narcotics requirements. Raymond et al. [2] found that the subjective quality of night sleep in hospitalized adult burn patients can significantly predict the person's pain intensity on the following day. An increased ratio of alpha waves during nonrapid eye movement (NREM) sleep and a decrease in the pressure pain threshold in fibromyalgia patients [3,4] and other chronic pain conditions [5,6] were reported but they failed to show specificity from healthy control subjects.

In human studies, there are no consistent findings in the change of pain sensitivity after sleep deprivation. Lentz et al [7] reported a decreased mechanical pain threshold in specific points after slow-wave sleep deprivation over two nights and Onen et al published the result that mechanical pain tolerance thresholds were decreased after total sleep deprivation. [8] In contrast, Arima et al reported that there were no effects of slow-wave sleep deprivation on pain thresholds in patients with the regional pain of temporomandibular disorder. [9]

In studies on rats, although majority of the experiment data are from study of REM sleep deprivation but their conclusions are much more consistent than those obtained from the humans. Under REM sleep deprivation in rats, nociceptive sensitivity to mechanical, [10] and electrical [11,12] stimuli was noted to have increased after the deprivation. Onen et al.[13] provided REM sleep deprivation for 3 consecutive days in Wistar rat and found that the deprivation induced decreased thresholds to thermal and mechanical stimuli but not to chemical stimuli (formalin test). Thus all REM sleep deprivation studies in rats consistently suggested that REM sleep deprivation increases nociceptive behavior. However, there was

no data on whether total sleep deprivation or deprivation of other stages of sleep such as NREM sleep may produce the same changes in nociceptive sensitivity.

It is known that pathophysiologies of total sleep deprivation was difficult to apprehend and it was only in the past 2 decades with the successful development of animal models for total sleep deprivation [14] that has permitted us to know the condition better. With support from the grant NSC-88-2314-B-182-090 we studied the relationship between sleep deprivation and nociception using tail-flick test for the measurement of pain threshold. In a randomized controlled cross over design, we subjected different intervals of sleep deprivation condition to 20 rats and demonstrated that after 1, 3, and 5 days of sleep deprivation, there were a significant increase from baseline in tail flick latency by 35%, 36% and 30% respectively. [15] With results from our work we felt that as tail flick is a test that measures reflex action through acute pain, experiment with Formalin test [16] which quantitates behavioral response to a relatively long lasting pain stimulus resembling pain as it is seen in actual disease states should review more information on the topic.

Recent studies have demonstrated that many immediate early genes, transcription factors and mitochrondrial genes are expressed at higher levels in painful stimulus. C-fos (an immediate early gene) immunoreactivity has been useful in constructing maps of post-synaptic neuronal activity with single cell resolution, and has been suggested to be tightly correlated with ongoing neuronal activity. In the past, numerous studies have successfully used c-fos immunoreactivity in formalin test to quantitate responses to pain in related to conditions that may affect or treat pain [17, 18, 19, 20, 21].

We have previously demonstrated that seven days of total sleep deprivation results in a 25% decrease of halothane minimal alveolar concentration in rats. [22,23] However, there is no reports in the literature concerning the effects of total sleep deprivation on c-fos expression and opiates requirement.

As we had previously demonstrated that total sleep deprivation affects pain threshold [15] and opiates modify induction of c-fos activity in the spinal cord of the rat following noxious stimulation and on a dose-dependent manner, [17, 18] we hypothesize that total sleep deprivation will affect the c-fos expression under painful stimulation.

Aim of the Study

The aim of this study was to assess the effect of total sleep deviation on the reaction of rats subjected to different noxious stimuli.

研 究 方 法

Materials and methods

Preparation of Animals

With approval by the Animal Use and Research Committee of the China Medical University Hospital, 52 Sprague-Dawley rats weighing 250-350 g were used for the study. Initially, each rat was anesthetized with intra-peritoneal ketamine (70 mg/kg) and xylazine (6 mg/kg). Five stainless steel screws (Small Parts Inc., Miami Lakes, FL) were implanted through the skull to serve as dural electroencephalographic electrodes. At the conclusion of surgery the rat was placed into its original cage to rest for 7 days. After 7 days of rest the rats were placed into the sleep deprivation apparatus for 3 days in order to adapt the laboratory condition before testing.

Sleep deprivation apparatus

Total sleep deprivation was elicited using the dish-over-water method with the Rechtschaffen apparatus (Fig. 1). The apparatus consisted of two plastic cages, each 60 cm (l) x 20 cm (w) x 60 cm (h), placed side by side, for the housing of two rats. A 40 cm diameter smooth circular plastic disc with its center in the alley between the cages and protruding 15.5 cm under each cage to provide a partial floor with approximately the same area as a home cage (492 cm^2) constitutes the ground the rats could stay. Beneath each side of the disc and extending beyond it to the walls of each cage was a tray of 2-3 cm deep water. The underside of the disc was attached to a computer control motor which can rotate the disc on demand. Cage temperature was regulated at 24-26 ºC; pan water was 3-4 degree lower. Drinking water and food will be available *ad libitum* from bottles and feeders hanging on the side of the side walls of the alley. The cage was maintained on a 12 hour light and 12 hour dark cycle (lights on at 08:00 am).

Figure 1, The Rechtschaffen Sleep Deprivation Model

Sleep deprivation procedure

At the start of sleep deprivation the electroencephalographic electrodes were attached to a computerized MP 150 (BIOPAC System, Inc.). Upon detecting a sleep state, the computer started the motor beneath the disc to rotate the disc in a counterclockwise direction at a slow rotation rate of 3 rev/min. As the disc was rotating, the rats were disturbed and had to walk opposite to the direction of rotation in order not to fall into the water (rats are hydrophobia). A special board was used to cover the rotary disc on the side where the control rat resided so that the disc movement will not disturb the rat on it. The whole design is such that both rats were subjected to the same laboratory condition except that the rats on the covered disc will be able to sleep, whereas rats on the rotary disc will not.

Study design

Two different experiments were performed to assess the sensitivity to thermal and chemical stimuli. To minimize changes due to circadian rhythm, [24] all sensitivity to noxious stimuli measurements were performed in the morning between 7:00 a.m. and 12:00 noon. In each experiment, the investigator was blinded to the type of sleep manipulation the rat had experienced.

Assessment of nociception

Experiment 1: tail flick test

For the thermal experiment, 16 SD male rats were randomly assigned into two groups: control (n=8), and total sleep deprivation rats (n=8). Each rat was randomly subjected to either one of the two different conditions for 24 hours: condition I, continuous enforced locomotion applied to the rat; condition II, adequate rest (the rats were left undisturbed). Group A rats experienced condition I first followed by condition II, whereas, group B rats experienced condition II first and then condition I. The rats are allowed to rest in situ for a period of five days in between the two conditions so that the rats can have enough rest in between study.

Tail-flick latency test (TFL, Ugo Basile, Milan, Italy) were performed and results recorded on the morning of day before and the second day after sleep deprivation. Each rat was placed in a cylindrical plastic restrainer (Broome Rodent Restrainer; Harvard Apparatus, South Natick, MA) for 30 minutes prior to measurement to allow them to get used to the restrainer. The restrainer prevented the rat to move freely but allowed easy access to the length of the tail. An intensity of heat was arranged so that baseline TFL occurred between 3 and 4 seconds. A cut off latency of 10 seconds was arbitrarily selected. Each TFL measurement consisted of a mean of three separate trials over 5 minutes. Values of pain threshold were expressed as percent of TFL changes from baseline and % maximal possible effect (%MPE) =

$$\frac{\text{TFL s/p condition} - \text{baseline TFL} \times 100}{\text{cut off time (10 second)} - \text{baseline TFL}}$$

This data expression normalizes the distribution of data while retaining the graded analysis. [25]

After recording of the tail flick responses, naloxone 1mg/kg i.p. was injected and tail-flick test were performed and measurements were recorded again 5 minutes later.

Experiment 2: formalin test

For chemical noxious stimuli, 36 rats were randomly allocated into 6 groups, group C1, C2, C3, D1, D2 and D3. After 3 days of adaptation period, rats from Group D1, D2 and D3 were subjected to 1, 2 and 3 days of sleep deprivation respectively. Group C1, C2, C3 rats acted as control and were given a condition that allowed adequate sleep for 1, 2 and 3 days respectively. Formalin test was applied to Group D1, D2 and D3 rats on the morning of the 2nd, 3rd, and 4th day after confinement. Group C1, C2, C3 rats had the test on the morning

immediately after the adequate sleep period.

All rats were placed in an observation chamber for 15 minutes before the formalin injection. The chamber was a 30 x30 x 30 cm Plexiglas box with a mirror below the floor at a 45 ºangle to allow an unobstructed view of the paws.

Formalin test began with a subcutaneous injection of 50μl of sterile 5% formalin into the rat's plantar surface of the left hind paw using a 1 ml syringe with a 26-G needle. The fluid was delivered as rapidly as possible while the rats were immobilized. Immediately after formalin injection, effects of formalin on overt behavior was assessed by observing spontaneous flinching and licking in rats for 60 min. Measurement of the behavior reaction was based on a scoring system described by Dubuisson and Dennis.[26, 27] The system divided behavior reaction into 4 categories: 0 = the injected paw was not favored (i.e. floot flat on floor with toes splayed); 1 = the injected paw has little or no weight on it with no toe splaying; 2 = the injected paw is elevated and the heel is not in contact with any surface; 3 = the injected paw is licked, bitten or shaken. The amount of time spent in each of the four behavior categories was recorded and calculated every minute by an anesthesiologist blinded to the treatment. At every 5 minutes intervals an average score was calculated and recorded. Results are expressed as mean score standard error of the mean (SEM) occurring in a given time period.

Within one hour after behavioral testing, rats were deeply anesthetized with ketamine (40~80 mg/kg body weight, i.p.). perfused intracardially with heparinized saline (1-2 min) followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4, Sigma). The complete spinal cord segments from L3-L6 where majority of sciatic afferent are, were removed. The segment was post-fixed in the same fixative for 12 h at 4 degree C and then transferred to a PBS containing sucrose (15-20%). The next day spinal cord segments were serially sectioned at 36-um thickness in a transverse plane with a freezing microtome. The sections were stained using a standard avidin-biotin-peroxidase complex (ABC) technique. Free-floating sections were incubated overnight in the primary rabbit antiserum directed against c-fos protein (Oncogene Res. Products) diluted 1:10,000 in PBS. After several washes in PBS the sections were incubated in biotinylated goat antirabbit IgG, diluted 1:200 in PBS and 3% normal goat serum for 2 h, rinsed in PBS and reacted with avidin-biotin reagents 1:100, for 1 h at room temperature. After three washes in Tri-buffered saline (TBS), sections were developed in diaminobenzidine tetrahydrochloride solution containing H_2O_2 in TBS. When a light background appeared, the reaction was stopped by four washes with PBS. Sections were then washed in distilled water, mounted on slides, air-dried, dehydrated through graded ethanol solutions followed by xylene and then coverslipped with Permount. The rats were sacrificed and c-fos immunoreactivity of the spinal cord was examined. Density of the labeled cells in the lumbar spinal laminae, conventionally grouped

into three groups (I-II, III-IV and V), was evaluated by a researcher unaware of the animal group assignment. Cell counts were made in all the processed section of each spinal cord at a final x40 magnification. The number of fos positive cells of each processed section of each spinal cord was divided by the number of counted tissue sections, in order to evaluate the average number of labeled cells for each rat.

Statistical analysis

Data are presented as mean standard error of the mean (S.E.M.). Values of TFL, percentage TFL changes from baseline, %MPE were compared between groups and analyzed by the Student's t-test to see if total sleep deprivation or naloxone effects on tail flick responses were significant. The data for behavior reaction and c-fos immunoreactivity of the spinal cord of rats in the formalin test were analyzed and compared between groups (control rats and total sleep deprived rats) by ANOVA followed by Dunnett's test. $P<0.05$ was regarded as significant for all comparison.

結　果

Results

Experiment 1: tail flick test

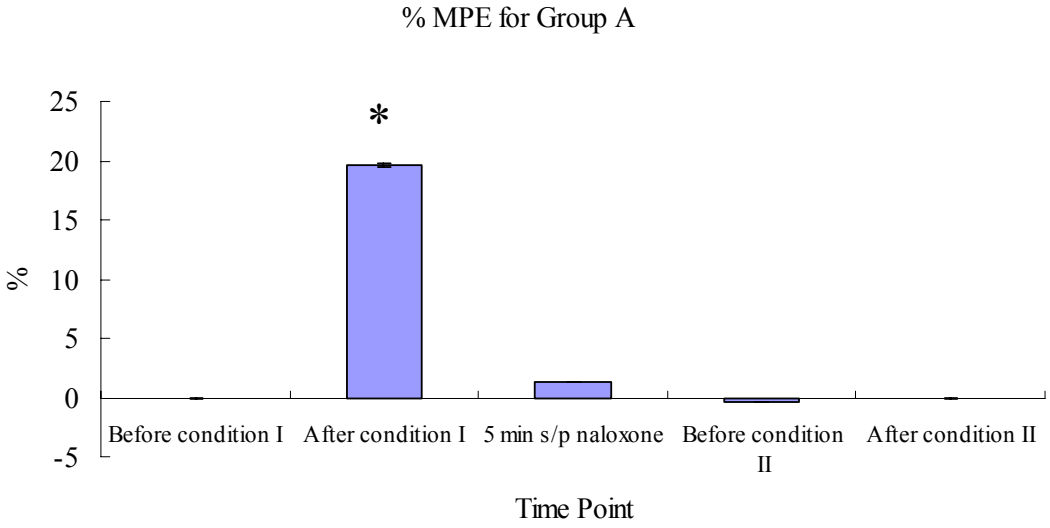
At baseline, the tail flick latency was not different between groups. After 24 h of total sleep deprivation, there were a significant increase ($p<0.01$) in tail flick latency. However, tail flick latency significantly decreased to near baseline value after naloxone injection (Table 1). Both changes were not significantly different between groups. The percentage TFL increase after 24 h of total sleep deprivation compared to value before conditioning was 33.4%±2.6% in group A rats and 27.0%±2.7% in group B rats. The percentage TFL decrease 5 min after naloxone injection compared to before injection was 23.3%±1.8% in group A rats and 21.0%±0.9% in group B rats. %MPE increased significantly whenever the rats were placed under sleep deprivation condition and returns to near baseline level after naloxone injection (Figure 1)

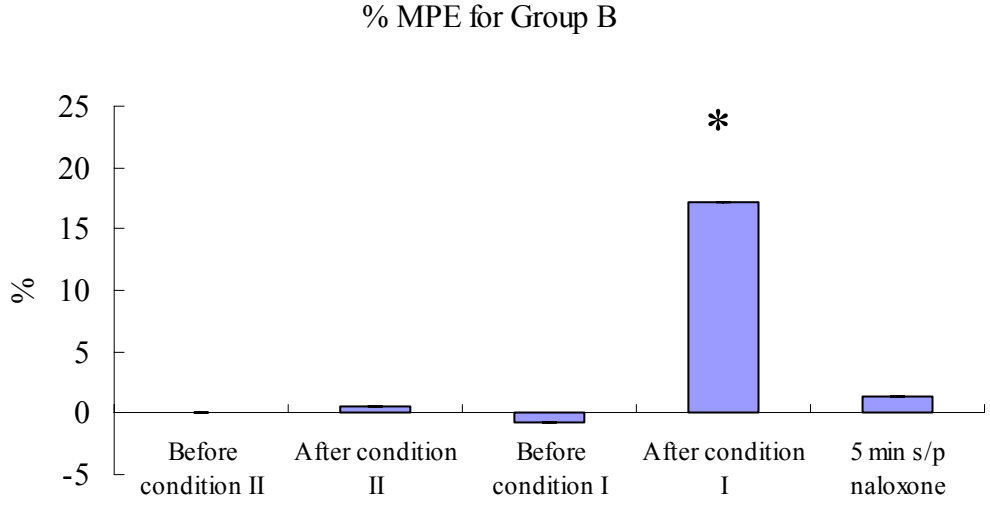
Table 1. Tail flick latency of Group A and B before conditioning, after 24 hours conditioning (condition I and II) and 5 min after naloxone injection.

Group	n	Condition I (sec)			Condition II (sec)	
		Before	s/p 24 hours	s/p naloxone	Before	s/p 24 hours
A	8	3.71±0.09	4.94±0.13*	3.79±0.14	3.69±0.09	3. 71±0.07
B	8	3.73±0.05	4.83±0.11*	3.82±0.09	3.78±0.06	3. 82±0.06

Values represents mean ± standard error of the mean
Condition I: sleep deprivation for 24 hours; condition II: adequate rest for 24 hours
* Differs from values on the day before conditioning and 5 min after naloxone injection; $p<0.01$, Student's t-test.

Figure 1. % MPE in Group A and Group B rats in time point before condition I, after condition I, after naloxone injection, before condition II and after condition II. Values are mean SEM; * : $p<0.01$ compared to before condition I and 5 min after naloxone injection





Experiment 2: formalin test

Nociceptive behaviors

Recording of flinching and licking, at 5 min intervals for 60 min post-injection in all rats clearly demonstrated a biphasic response (*Figure 2*). There was no significant difference in the mean duration of phase 1 and phase 2 nociceptive behaviors between Group C1, C2, C3, D1, D2, and D3 rats.

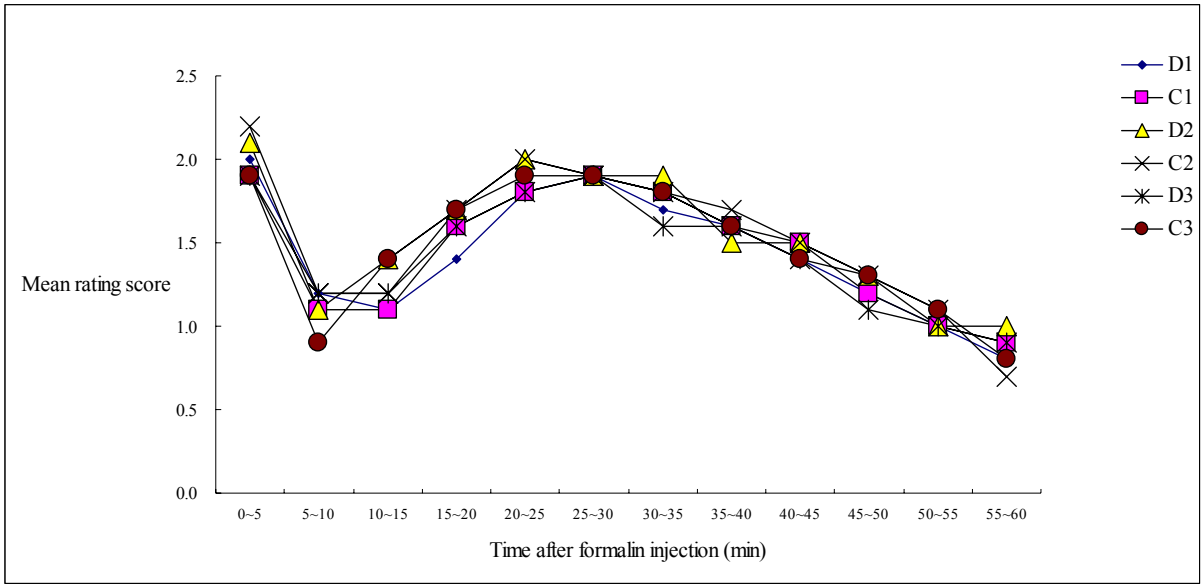


Figure 2. Time course of behavior response to hindpaw formalin injection. Each data point represents the mean rating score of a particular time period. D1, D2 and D3 represented data from rats in sleep deprivation enviroment for 24h, 48h and 72 h respectively. C1, C2, and C3 represented data from rats in adequate sleep environment for 24h, 48h and 72 h respectively

c-fos immunoreactivity

Number of c-fos-like immunoreactive neurons was significantly increased in the dorsal horns of rats that had total sleep deprivation for 48 h and 72 h compared with adequate sleep environment (Figure 3). This increase was observed in the Superficial (I-II), Nucleus Proprius (III-IV) and Neck (V) lamina of the dorsal horn. (Table 2 & Figure 4)

Figure 3. Appearance of spinal cord slice illustrating fos-like immunoreactivity at the L4-5 spinal segment ipsilateral to the hindpaw injection of formulin. Four experimental situations are represented: control (A); total sleep deprivation for 24 hours (B); total sleep deprivation for 48 hours (C); and total sleep deprivation for 72 hours (D). Number of c-fos-like immunoreactive neurons was significantly increased in the dorsal horns of rats that had total sleep deprivation for 48 h and 72 h

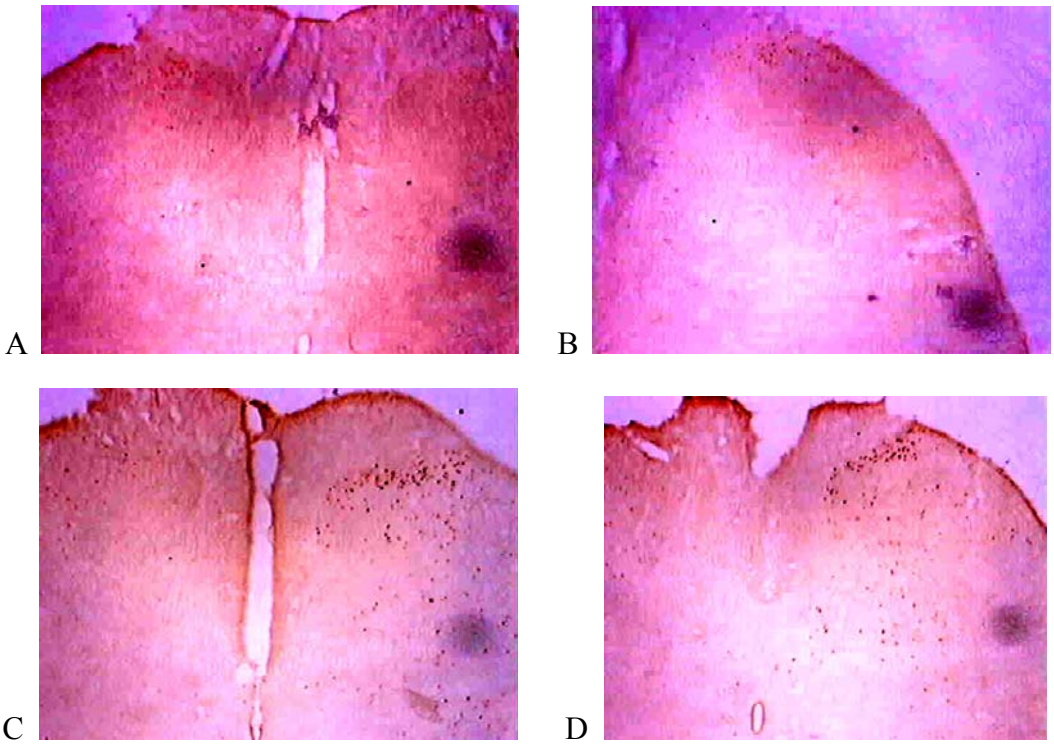


Table 2. Number of c-fos-like immunoreactive neurons was significantly increased in the dorsal horns of rats that had total sleep deprivation for 48 h and 72 h compared with adequate sleep environment. This increase was observed in the Superficial (I-II), Nucleus Proprius (III-IV) and Neck (V) lamina of the dorsal horn.

* & ǂ represents $p < 0.05$ compared with control

time	Sleep Deprivation (number of positive c-fos neuron per section)				Adequate Sleep (number of positive c-fos neuron per section)			
	Lamina I-II	Lamina III-IV	Lamina V	Total	Lamina I-II	Lamina III-IV	Lamina V	Total
24 h	13.4±3.6	7.8±2.2	9.6±2.3	10.3±1.6	13.5±3.6	8.8±2.5	10.7±3.0	11.0±1.7
48 h	52.8±7.3*ǂ	33.0±4.8*ǂ	42.4±4.5*ǂ	43.0±3.6*ǂ	28.3±2.8ǂ	16.0±1.8ǂ	21.5±2.0ǂ	22.0±1.7ǂ
72 h	49.5±2.8*ǂ	29.5±2.2*ǂ	37.1±2.5*ǂ	38.7±2.4*ǂ	20.8±2.2ǂ	11.7±1.4ǂ	15.6±2.0ǂ	16.0±1.4ǂ

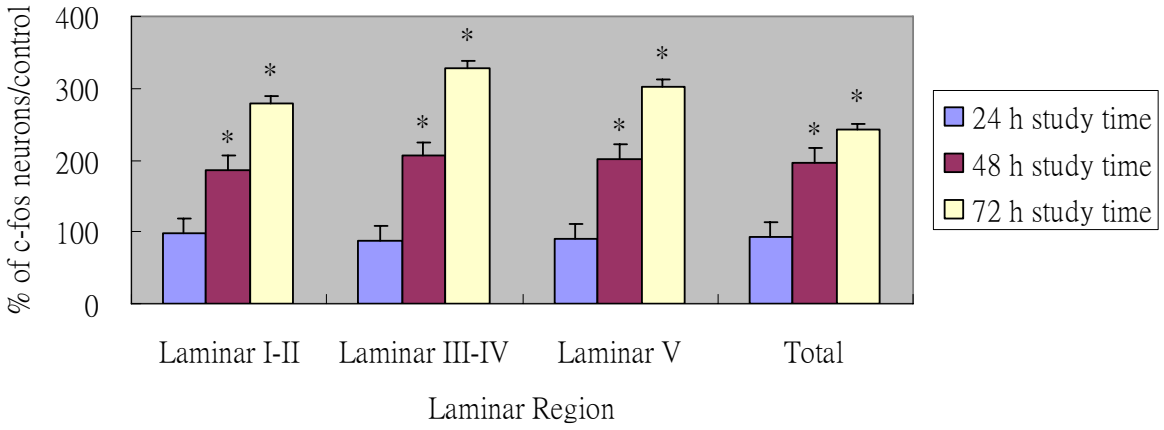


Figure 4. Effects of 24 h, 48 h, and 72 h of sleep deprivation on the expression of c-fos neurons in each laminar region (mean, SEM percentage control). Ratio of c-fos neurons in sleep deprivation group to control group plotted at each lamina: Laminar I-II = Superficial Laminar, Laminar III-IV = Nucleus Proprius, Laminar V = Neck of the Dorsal Horn. * represented $p<0.01$ compared with control.

討　論

Adequate sleep has been reported to help wound healing,[28] but contrary to it, sleep deprivation may lead to death through deterioration of body tissue and increase in the ratio of catabolism to anabolism. [29] Furthermore, sleep deprivation may also induce enormous physiological changes such as disturbed liver functions, serum lipid levels and hyperphosphatemia,[30] alteration in endocrine and hormonal functions, [31,32] leukocytosis and increases in natural killer activity,[33] decrease in body temperature and loss of body weight. Frequently, there are patients who are arriving for anesthesia or having pain have previous night/s sleepless. Patients coming from the ICU with no sedation during their previous ICU stay [34] and patients who are too anxious or too painful to be able to sleep are among the many examples. As sleep deprivation generates enormous physiological changes,[35] it will be valuable to know how it may influence anesthesia and the management of pain.

Firstly, it was reported in the literature that inhalation anesthetic agents influence sleep after anesthesia [36, 37]. However, there was no report on anesthetic requirement of patients with inadequate or complete deprivation of sleep. We understand that minimum alveolar concentration (MAC) for volatile anesthetics is influenced by many factors such as alcoholism, aging, sex, temperature, neurotransmitter release, hypocarbia and hypercarbia, metabolic and electrolyte imbalance, hypotension and hypertension, thyroid dysfunction, and pregnancy. [38, 39] Supported by the grant NSC-85-2331-B182-007 and grant NSC-87-2314-B182-048, we used total sleep deprivation (TSD) in rats as a model to test the hypothesis that TSD affects MAC of halothane.[22,23] In the first experiment, forty rats subjected to seven days of sleep deprivation were given inhalation anesthesia and their MAC measured. We demonstrated that following seven days of TSD, body weight decreased by 13% and halothane MAC decreased by 25% for all rats and there was 5 death during anesthesia [29]. Then, we examined the changes in MAC of halothane in rats with different sleep deprivation intervals. We demonstrated that after 1, 3, and 5 days of sleep deprivation, there were decreases from baseline MAC by 12%, 12% and 16% respectively（$P<0.05$ vs before deprivation）.[30] Our results agree with results from Tung et al. which were published after we concluded our study. They demonstrated that TSD potentiates the loss of righting reflex induced by propofol and isoflurane. [40, 41] These results might be of immediate clinical importance to humans who are suffering from profound sleep disturbance before anesthesia. Our result showed that adjustments and titration in anesthetic dosage may be necessary for such patients. The results also urge us to investigate into the relationship between TSD and nociception.

With the results from our work we felt that as TSD generates enormous physiological changes [35] and it also affects inhalation anesthetic MAC, it should be interesting to know if TSD could

affect nociception.

During that time, relationship between sleep and pain was only supported by correlated findings. Morin et al.[1] described that pain may disturb or interrupt sleep as their chronic pain patients frequently suffered from sleep disturbances, however, sleep disturbances may also influence pain intensity and narcotics requirements. Raymond et al.[2] found that the subjective quality of night sleep in hospitalized adult burn patients can significantly predict the person's pain intensity on the following day.

Findings in other human studies on the change of pain sensitivity after sleep deprivation are rather controversial. Lentz et al [7] reported a decrease in mechanical pain threshold in specific points after slow-wave sleep deprivation for over two nights. Onen et al [8] published that mechanical pain tolerance thresholds were decreased after total sleep deprivation. In contrast to those studies, Arima et al [9] reported that there were no effects of slow-wave sleep deprivation on pain thresholds in patients with regional pain of temporomandibular disorder. Older et al [42] also found that there was no difference in pain threshold in healthy subjects with slow wave sleep deprivation for 3 consecutive nights vs. untreated control.

It is interesting that results on rats in the effects of REM sleep deprivation on pain sensitivity are rather consistent. In several studies it was reported that nociceptive sensitivity to mechanical,[10] and electrical stimuli[11,12] were increased after REM deprivation. Onen et al. [13] provided REM sleep deprivation for 3 consecutive days in Wistar rat and found that the deprivation induced decreased thresholds to thermal and mechanical stimuli but not to chemical stimuli (formalin test). The REM sleep deprivation data reported here suggest that REM sleep deprivation increases nociceptive behavior in rats. However, there was no data on TSD or deprivation of other stages of sleep to show whether these conditions may produce the same changes in nociceptive sensitivity.

With support from the grant NSC-88-2314-B-182-090 we studied the relationship between TSD and nociception using tail-flick test for the measurement of pain threshold. In a randomized controlled cross over design, we subjected different intervals of TSD condition to 20 rats and demonstrated that after 1, 3, and 5 days of sleep deprivation. The result demonstrated that there were a significant increase from baseline in tail flick latency (TFL) by 35%, 36% and 30% respectively. [15] This result contradicts the previous findings on REM sleep deprivation but agrees with our previous finding from the MAC (measured by the induction of a noxious stimulation over the tail of a rat) of inhalation anesthetic agent that less inhalation anesthetic is needed to anesthetize rats after TSD.

As tail flick test only measures transient pain, the mechanism of TSD influencing pain perception could not be revealed merely with the result. It is known that the Formalin test quantitates the behavioral response to a relatively long lasting pain stimulus, which resembles pain as it is seen in actual disease states.[16] Recent studies have demonstrated that many

immediate early genes, transcription factors and mitochrondrial genes are expressed at higher levels in painful stimulus. c-fos (an immediate early gene) immunoreactivity has been useful in constructing maps of post-synaptic neuronal activity with single cell resolution, and has been suggested to be tightly correlated with ongoing neuronal activity. In the past, numerous studies have successfully used c-fos immunoreactivity in formalin test to quantitate responses to pain in related to conditions that may affect or treat pain [17, 18, 19, 20, 21].

As TSD attenuates halothane minimal alveolar concentration in rats [16, 17] and augments pain threshold [18] we hypothesize that TSD will affect the c-fos expression under the noxious stimulation condition from the Formalin test.

With the support from this grant NSC-92-2314-B-039-012 we investigated further on the mechanism of how TSD affects nociception. With a blind randomized controlled design we performed 2 experiments. First we hypothesized that if effect of TSD on TFL can be antagonized by naloxone (a morphine antagonist), then TSD probably works through the morphine receptor pathway in smoothing pain. Thus, we reproduced our previous tail flick experiment and use naloxone to treat rats that had been subjected with TSD condition. Our result showed that TSD does prolonged TFL but TFL returned to original baseline after naloxone injection proving that TSD affects pain through the morphine pathway.

Then we performed our second experiment to study the effects of TSD on behavior reaction and c-fos immunoreactivity of spinal cord in rats after subcutaneous formalin injection. In this study we found that formalin test pain behavior was not changed after sleep deprivation versus adequate sleep but the number of c-fos-like immuno-reactive neurons was significantly increased in the dorsal horns of rats that had TSD. This augmentation was observed in the superficial (I-II), nucleus proprius (III-IV) and deep (V) lamina of the dorsal horn meaning that TSD affects nociceptive perception in a more complex manner than REM sleep deprivation.

Our result is important as previously there were no results on the effects of TSD on pain threshold in rats and we have demonstrated that the effect of TSD on nociception is different from that of REM sleep deprivation. We do not have enough information in the literature to explain why in one hand TSD prolongs TFL and decreases inhalation anesthetic MAC but on the other hand increases the number of c-fos positive neurons. However in the near future, we are planning to use a densometry to measure the quantity of neurons that have functional c-fos expression in the hope to know exactly if the increased in number signifies a real nociceptive event.

參 考 文 獻

References:

1. Morin CM, Gibson D, Wade J. Self-reported sleep and mood disturbance in chronic pain patients. Clin J Pain 1998;14:311-314.
2. Raymond I, Nielsen TA, Lavigne G, Manzini C, Choiniere M. Quality of sleep and its daily relationship to pain intensity in hospitalized burn patients. Pain 2001;92:381-388.
3. Moldofsky H, Scarisbrick P, England R, Smythe H. Musculoskeletal symptoms and non-REM sleep disturbance in patients with “fibrositis syndrome” and healthy subjects. Psychosom Med 1975;37:341-51.
4. Agargun MY, Tekeoglu I, Gunes A, Adak B, Kara H, Ercan M. Sleep quality and pain threshold in patients with fibromyalgia. Compr Psychiatry 1999;40:226-8.
5. Drewes AM, Svendsen L, Taagholt SJ, Bjerregard K, Nielsen KD, Hansen B. Sleep in rheumatoid arthritis: A comparison with healthy subjects and studies of sleep/wake interactions. Br J Rheumatol 1998;37:71-81.
6. Schneider-Helmert D, Whitehouse I, Kumar A, Lijzenga C. Insomnia and alpha-sleep in chronic non-organic pain as compared to primary insomnia. Neuropsychobiology 2001; 43:54-8.
7. Lentz MJ, Landis CA, Pothermel J, Shaver JL. Effects of selective slow wave sleep disruption on musculoskeletal pain and fatigue in middle age women. J Rheumatol 1999;26:1586-92.
8. Onen SH, Alloui A, Gross A, Eschallier A, Dubray C. The effects of total sleep deprivation, selective sleep interruption and sleep recovery on pain tolerance thresholds in healthy subjects. J Sleep Res 2001;10:35-42.
9. Arima T, Svensson P, Rasmussen C, Nielsen KD, Drewes AM, Arendt-Nielsen L. The relationship between selective sleep deprivation, nocturnal jaw-muscle activity and pain in healthy men. J Oral Rehabil 2001;28:140-8.
10. Onen SH, Alloui A, Eschallier A, Dubray C. Vocalization thresholds related to noxious paw pressure are decreased by paradoxical sleep deprivation and increased after sleep recovery in rat. Neurosci Lett 2000;291:25-8.
11. Hicks RA, Moore JD, Findley P, Hirshfield C, Humphrey V. Sleep deprivation and pain thresholds in rats. Percept Mot Skills 1978;47:848-50.
12. Hicks RA, Coleman DD, Ferrante F, Sahatjian M, Hawkins J. Pain thresholds in rats

during recovery from REM sleep deprivation. Percept Mot Skills 1979;48:687-90.

13. Onen SH, Alloui A, Jourdan D, Eschallier A, Dubray C. Effects of rapid eye movement (REM) sleep deprivation on pain sensitivity in the rat. Brain Res 2001;900:261-7.
14. Rechtscaffen A, Bergmann BM. Sleep deprivation in the rat: an update of 1989 paper. Sleep 2002;25(1):18-24.
15. ***Wu RSC***, Liu SJ, Wu KC, Cheng FC, Hwang TL, Hsu JC, Tan PC. Effects of Sleep Deprivation on Pain Threshold in Rats. 國科會NSC-87-2314-B182-048 報告.
16. Dubuisson D, Dennis SG. The formulin test: A quantitative study of the analgesic effects of morphine, merperidine, and brain stem stimulation in rats and cats. Pain 1977;4:161-174.
17. Tolle TR. Castro-Lopes JM. Coimbra A. Zieglgansberger W. Opiates modify induction of c-fos proto-oncogene in the spinal cord of the rat following noxious stimulation. Neuroscience Letters 1990;111(1-2):46-51.
18. Presley RW. Menetrey D. Levine JD. Basbaum AI. Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. Journal of Neuroscience 1990;10(1):323-35.
19. Fukuda T. Nishimoto C. Shiga Y. Toyooka H. The formalin test: effects of formalin concentration and short-term halothane inhalation. Regional Anesthesia & Pain Medicine 2001;26(5):407-13,.
20. Fukuda T. Nishimoto C. Miyabe M. Toyooka H. The residual effects of hemorrhagic shock on pain reaction and c-fos expression in rats. Anesthesia & Analgesia 2003;93(2):424-9.
21. Bester H. Beggs S. Woolf CJ. Changes in tactile stimuli-induced behavior and c-Fos expression in the superficial dorsal horn and in parabrachial nuclei after sciatic nerve crush. Journal of Comparative Neurology. 2000;428(1):45-61.
22. ***Wu RSC***, Liu SY, Wu KC, Wong CL, Tan PC. Sleep deprivation influences halothane minimum alveolar concentration in rats. 國科會NSC-85-2331-B182-007 報告.
23. ***Wu RSC***, Liu SY, Wu KC, Wong CL, Tan PC. The effects of different sleep deprivation intervals on halothane MAC in rats. 國科會NSC-88-2314-B182-090 報告.
24. Munson ES, Martucci RW, Smith RW. Circadian variations in anesthetic requirement and toxicity in rats. Anesthesiology 1970;32: 507-14.
25. Fender C, Fujinaga M, Maze M. Strain Differences in the Antinociceptive Effect of Nitrous Oxide on the Tail Flick Test in Rats. Anesth Analg 2000;90(1):195-199.
26. Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of

morphine, meperidine, and brain stem stimulation in rats and cats. Pain 1977;4:161-174.

27. Sun WZ, Shyu BC, Shieh JY. Nitrous oxide or halothane, or both, fail to suppress c-fos expression in rat spinal cord dorsal horn neurones after subcutaneous formalin. British Journal of Anaesthesia 1996;76:99-105.
28. Adam K, Oswald I: Sleep helps healing. British Medical Journal 1984;289(24): 1400-1401.
29. Rechtschaffen A, Gilliland MA, Bergmann BM, Winter JB. Physiological correlates of prolonged sleep deprivation in rats. Science 1983;221:182-184.
30. Ilan Y, Martinowitz G, Abramsky O, Glazer G, Lavie P. Prolonged sleep deprivation induced disturbed liver functions, serum lipid levels, and hyperphosphatemia. European Journal of Clinical Investigation 1992;22(11):740-743.
31. Baumgartner A, Dietzel M, Saletu B, Wolf R, Campos-Barros A, Graf KJ, Kurten I, Mannsmann U. Influence of partial sleep deprivation on the secretion of thyrotropin, thyroid hormones, growth hormone, prolactin, luteinizing hormone, follicle stimulating hormone, and estradiol in healthy young women. Psychiatry Research 1993; 48(2):153-178.
32. Szuba MP, Altshuler LL, Baxter LR Jr. Thyroid function and partial sleep deprivation response. Archives of General Psychiatry 1992;49(7):581-582.
33. Dinges DF, Douglas SD, Zaugg L, Campbell DE, McMann JM, Whitehouse WG, Orne EC, Kapoor SC, Icaza E, Orne MT. Leukocytosis and natural killer cell function parallel neurobehavioral fatigue induced by 64 hours of sleep deprivation. Journal of Clinical Investigation 1994;93(5):1930-1939.
34. Puntilio KA. Pain experience of ICU patients. Heart Lung 1990;19:526.
35. Rechtschaffen A, Bergmann BM, Everson CA, Kushida CA, Gilliland MA. Sleep deprivation in the rat. Sleep 1989;12 (1):1-21.
36. Moote CA, Knill RL. Isoflurane anesthesia causes a transient alteration in nocturnal sleep. Anesthesiology 1988;69:327-331.
37. Knill RL, Skinner MI, Novick T, Vandenberghe HM, Moote CA. The night of intense REM sleep after anesthesia and surgery increases urinary catecholamines. Canadian Journal of Anaesthesia 1990;37:S12.
38. White PF, Johnson RR, Eger EI II. Determination of anesthetic requirement in rats. Anesthesiology 1974;40:52-57.
39. Quasha AL, Eger EI II, Tinker JH: Determination and applications of MAC. Anesthesiology 1980;53:315-334.

40. Tung A, Szafran MJ, Bluhm B, Mendelson WB. Sleep deprivation potentiates the onset and duration of loss of righting reflex induced by propofol and isoflurane. Anesthesiology 2002;97:906-11.

41. Tung A, Herrera S, Szafran MJ, Kasza K, Mendelson WB. Effect of sleep deprivation on righting reflex is partially reversed by administration of adenosine A1 and A2 receptor antagonist. Anesthesiology 2005;102:1158-1164.

42. Older SA, Battafarano DF, Danning CL. The effects of delta wave sleep interruption on pain thresholds and fibromyalgia-like growth factor I. J Rheumatol 1999;26:1586-1592.

計畫成果自評

本計畫內容與原計畫相符程度：達 80%。

達成預期目標情況：
預期目標一：我們曾在之前的研究中發現缺乏睡眠(Total sleep deprivation；TSD)會降低大白鼠吸入性麻醉劑之最低肺泡濃度，故認為 TSD 也會影響疼痛閾值。又倘若 TSD 影響疼痛閾值之機轉是經鴉片途徑的，則像 Naloxone 一類的嗎啡拮抗劑，將可拮抗 TSD 對疼痛閾值的效應。在本實驗一「Naloxone 對缺乏睡眠提升大白鼠疼痛閾值的效應」中，我們的實驗結果顯示閾值提高擺尾測驗的疼痛閾值，而且施打 naloxone 後，讓 TSD 所導致的高疼痛閾值會因施打 naloxone 而降回到原先之基礎值上。表示 TSD 會影響疼痛閾值，而 TSD 是經鴉片途徑之機轉來影響疼痛閾值。
預期目標二：研究既然 TSD 會影響擺尾測驗的疼痛閾值，則也應該對大白鼠皮下注射福馬林後，所產生之疼痛行為反應及脊髓背角 c-fos 活動表現上有所影響。在本實驗二「缺乏睡眠對大白鼠皮下注射福馬林後行為反應及脊髓背角 c-fos 活動表現上的效應」中，我們的實驗結果顯示 TSD 24hr，48hr 及 72hr 雖對大白鼠皮下注射福馬林後的行為反應上無明顯的影響，但在脊髓背角 c-fos 活動的表現上，則在 48hr 及 72hr 上有明顯的增加反應。表示 TSD 會確實影響大白鼠皮下注射福馬林後之表現。

研究成果之學術或應用價值：因為目前醫學文獻睡眠剝奪對疼痛之相關性研究中，都是以缺乏快速動眼睡眠(REM Sleep Deprivation; REMSD)為主。TSD 對疼痛之相關性研究則非常缺乏。其他文獻資料顯示 REMSD 會降低疼痛閾值，而本研究發現在 TSD 剝奪之下，疼痛閾值不但沒有下降，反而是上升之現象。這表示我們是首先發現了 TSD 與 REMSD 在影響疼痛上的不同，是對瞭解睡眠上有更進一步的認知，故本研究成果應該在學術研究上具有一定之應用價值。