CHATER III

MATERIALS AND METHODS

ANIMALS

Male Wistar rats (200-300 g) were purchased from the National Animal Center at Salaya, Mahidol University Salaya Campus, Thailand.

CHEMICALS

1. Animal Preparations

- di-ethyl-ether was purchased from Merck, Darmstadt, Germany.
- Normal saline was purchased from Hospital Products Public Co, Ltd.
- Pento bartital Sodium (Nembutal®) was purchased from Sanofi (Thailand) Ltd.

2. Drugs

- Complete Freund's adjuvant (CFA) was purchased from Sigma, Steinheim, Germany.
- Ketanserin tartrate was purchased from Sigma, Steinheim, Germany.
- Para-chlorophenylalanine (PCPA) was purchased from Sigma, Steinheim, Germany

3. Immunohistochemical Study

- 3 3′-Diaminobenzidine (DAB) was purchased from DAKO, Carpentaria, CA, USA.
- *c-fos* primary antibody was purchased from Santa Cruz, USA.
- Envision labelling, HRP anti-mouse anti-rabbit was purchased from DAKO, Carpentaria, CA, USA.
- Hydrogen peroxide (H_2O_2) was purchased from Riedel-de Hach, Germany.
- Normal horse serum was purchased from DAKO, Carpentaria, USA.
- Sodium chloride (NaCl) was purchased from Merck, Darmstadt, Germany.
- Triton-X100 was purchased from Sigma, Steinheim, Germany.

METHODS

EXPERIMENTAL ANIMALS

Adult male Wistar rat weighing 200-300 grams were used in this study. They were allowed to rest for a week after arrival at the Animal Center, Faculty of Medicine, Chulalongkorn University before used in the experiment. The animals were kept in a well ventilated room where temperature is 25 $\pm$ 3 °C. Rats were housed in stainless-steel cages with an automatic lighting schedule which provided darkness from 6.00 PM to 6.00 AM. All animals were allowed to access of dry rat chow and tap water *ad libitum*.

To reach the goal of this research, the experiments were separated into 2 parts as follows.

Part 1 To study the role of 5-HT_{2A} receptor in chronic pain model and the development of chronic pain state

In this part the experiment were further subdivided into 2 experiments

1.1 To study the effect of CFA-induced inflammation on rats' behaviors and Fos protein expression

1.2 To study the role of 5-HT_{2A} receptor in chronic pain model by using behavioral assessment and Fos protein expression

Part 2 To study the role of 5-HT_{2A} receptor in 5-HT depleted state on the changes of pain sensation

In this part the experiment were subdivided into 2 experiments

2.1 To study the effect PCPA-induced 5-HT depletion on rats' behaviors and Fos protein expression

2.2 To study the role of 5-HT_{2A} receptor in 5-HT depleted state by using behavioral assessment and Fos protein expression

EXPERIMENT 1 To study the role of 5-HT_{2A} receptor in chronic pain model and the development of chronic pain state

EXPERIMENT 1.1 To study the effect of CFA-induced inflammation on rats' behaviors and Fos protein expression

In this study, the experimental animals were divided into 2 groups. Groups were comprised:

(1) Control group (n=25): Rats were were injected with 0.05 ml of normal saline (NSS) into their right hind paws.

(2) Peripheral inflammation groups (n=25): Rats were injected 0.05 ml of Complete Freund's Adjuvant (CFA) (Okamoto et al., 2002) into their right hind paws.

The animals were subdivided into 5 subgroups in order to study the development of chronic pain state at 0 hour (D0 groups), 1 day, 3 days, 5 days, and 7 days after CFA injection respectively.

Two hours after video recording, the rats were evaluated for the thermal hyperalgesia by the paw withdrawal test. After that, the rats were sacrificed then their brains were removed for Fos protein immunohistochemical study.

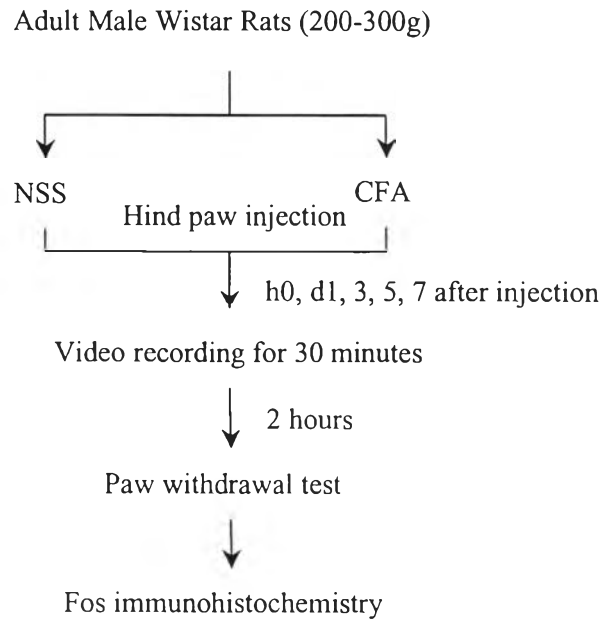

Figure [3-1] To study the effect of CFA-induced inflammation on rats' behaviors and Fos protein expression

EXPERIMENT 1.2 To study the role of 5-HT_{2A} receptor in chronic pain model by using behavioral assessment and Fos protein expression

In this experiment, 5-HT_{2A} receptor antagonist, ketanserin study was performed. Day 3 groups in experiment 1 were selected to study the role of 5-HT_{2A} receptor antagonist on nociceptive behavior.

Rats were administered intraperitoneally with 0.3 mg/kg of ketanserin in the volume of 0.2-ml/100 g body weight. The results of these groups were compared with the Day 3 group of experiment 1.1

(1) Control group with ketanserin treated (n=5): Rats were injected 0.05 ml of NSS into their right hind paw. One hour prior to video recording, rats were administered with 0.3 mg/kg body weight of ketanserin intraperitoneally in the volume of 0.2-ml/100 g body weight. Four hours after first ketanserin injection, rats were administered again with ketanserin at the same dose as first injection. One hour later, rats were anesthetized with sodium pentobarbital, 25 mg/kg body weight. Then the paw withdrawal test was started.

(2) Peripheral inflammation groups with ketanserin treated (n=5): Rats were injected 0.05 ml of CFA into their right hind paw. One hour prior to video recording, rats were administered with 0.3 mg/kg body weight intraperitoneally in the volume of 0.2-ml/100 g body weight. Four hours after first ketanserin injection, rats were administered again with ketanserin at the same dose as first injection. One hour later, rats were anesthetized with sodium pentobarbital, 25 mg/kg body weight. Then the paw withdrawal test was started

After finishing the experiments, the rats were sacrificed then their brains were removed for Fos immunohistochemical study

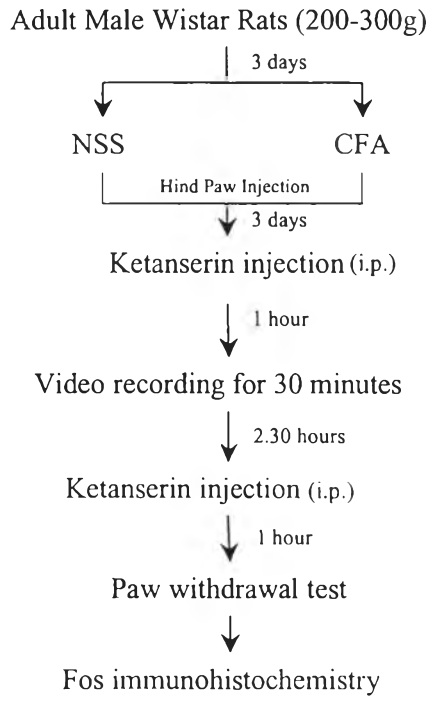

Figure [3-2] To study the role of 5-HT_{2A} receptor in chronic pain model by using behavioral assessment and Fos protein expression

EXPERIMENT 2 To study the role of 5-HT_{2A} receptor in 5-HT depleted state on the changes of pain sensation

EXPERIMENT 2.1 To study the effect PCPA-induced 5-HT depletion on rats' behaviors and Fos protein expression

In this study, the experimental animals were divided into 2 groups. Groups were comprised:

(1) Control (normal 5-HT animals) (n=25): Rats were administered intraperitoneally with 100-mg/kg-bodyweight of PBS in the volume of 0.2 ml/100 g body weight. Three days after, rats were injected 0.05 ml of NSS into their right hind paw.

(2) Low 5-HT animals (n=25): Rats were administered intraperitoneally with 100 mg/kg of para-chlorophenylalanine (PCPA) in the volume of 0.2-ml/100 g bodyweight. Three days after, rats were injected 0.05 ml of NSS into their right hind paw.

The animals were subdivided into 5 subgroups in order to study the effect of inflammation at 0 hour, 1 day, 3 days, 5 days, and 7 days after NSS injection respectively.

Two hours after video recording, the rats were evaluated for the thermal hyperalgesia by the paw withdrawal test. After that, the rats were sacrificed then their brains were removed for Fos protein immuno-histochemical study.

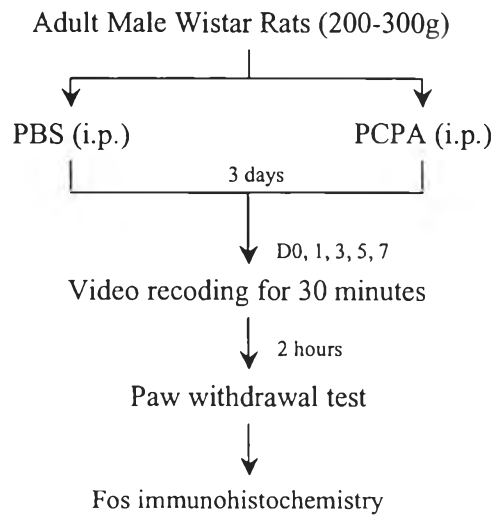

Figure [3-3] To study the effect PCPA-induced 5-HT depletion on rats' behaviors and Fos protein expression

EXPERIMENT 2.2 To study the role of 5-HT_{2A} receptor in 5-HT depleted state by using behavioral assessment and Fos protein expression

In this experiment, 5-HT_{2A} receptor antagonist, ketanserin study was performed. Day 3 groups in experiment 1 were selected to study the role of 5-HT_{2A} receptor antagonist on nociceptive behavior.

Rats were administered intraperitoneally with 0.3 mg/kg of ketanserin in the volume of 0.2-ml/100 g body weight. The results of this group were comparing to the low serotonin level rats in experiment 2

(1) Low 5-HT animals (n=5): Rats were administered intraperitoneally with 100 mg/kg of para-chlorophenylalanine (PCPA) in the volume of 0.2-ml/100 g bodyweight. Three days after, rats were injected 0.05 ml of NSS into their right hind paw.

(2) Low 5-HT animals with ketanserin treated (n=5): Rats were administered intraperitoneally with 100-mg/kg-bodyweight of PCPA in the volume of 0.2 ml/100 g body weight. Three days after, rats were injected 0.05 ml of NSS into their right hind paw. One hour prior to video recording, rats were administered with 0.3 mg/kg body weight intraperitoneally in the volume of 0.2-ml/100 g body weight. Four hours after first ketanserin injection, rats were administered again with ketanserin at the same dose as first injection. One hour later, rats were anesthetized with sodium pentobarbital, 25 mg/kg body weight. Then the paw withdrawal test was started.

After finishing the experiments, the rats were sacrificed then their brains were removed for Fos immunohistochemical study.

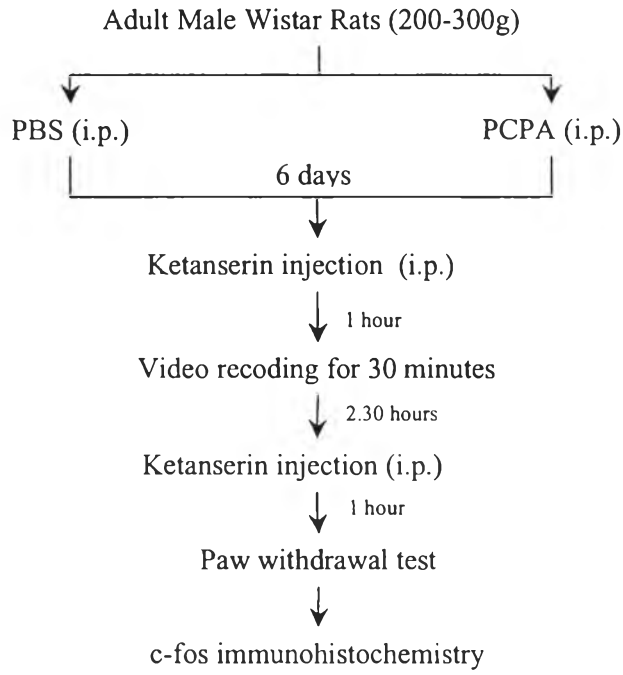

Figure [3-4] To study effect of 5-HT_{2A} receptor antagonist, ketanserin on observed behaviors in 5-HT depleted state and Fos expression

Induction of Serotonin depleted state

To induce serotonin depleted state in rats, PCPA was administered intraperitoneally. PCPA irreversibly inhibits tryptophan hydroxylase that acts as a rate-limited step in 5-HT synthesis, presumably by incorporating itself in to the enzyme to produce an inactive protein. This results in a long-lasting reduction of 5-HT levels. Recovery of enzyme activity and 5-HT biosynthesis requires the synthesis of new enzyme. (Frazer and Hensler, 1999).

In this study, PCPA was first dissolved in few drops of absolute ethyl alcohol and then phosphate buffer saline (PBS) was introduced in volume 0.2-ml/100 g-bodyweight. The dosage of PCPA for each experimental animal was 100 mg/kg bodyweight. This dosage of PCPA resulted in 75% depletion of 5-HT level in the body (Curzon and Green, 1970).

Three days after induction, the animals were used in further experiments (Sjoerdsma et al., 1970)

Induction of Inflammation

To induce inflammation, rats were anesthetized by diethyl ether. Each rat was subcutaneously injected with 0.05 ml of completed Freund's Adjuvant (CFA; Mycobacterium tuberculosis) into the right hind paw using micro syringe.

Preparation of ketanserin

In this study, ketanserin tartrate was dissolved in 3% DMSO.

Nociceptive Behavioral Study

The behavioral measurement in this research was separate to two test bases of pain.

1) The used of long-duration of noxious stimuli to study the changes of overall behaviors of the rats (tonic pain model)
2) The used of short-duration of noxious stimuli to evaluate the thermal pain responses (phasic pain model)

Observed behavioral study

To assess experimental animals' behaviors, they were placed in the observation chamber for measurement of their behaviors that will describe below. The observation chamber was placed with 2 windows of mirrors in the back of the chamber. The mirrors were placed in the anger of 60^{O}.It made the observer can see every position of the rats. Animals' behaviors were continuously recorded for 30 minutes using video system for playback analysis.

In this study, behaviors of the rats were classified into 4 categories.

1. Non-nociceptive behavior

Behaviors in this category comprised of (1) scratching their face (2) scratching their body, using un-injured hind paw (3) exploring

2. Nociceptive behavior

Behaviors in this category comprised of

a) Flinching - shaking the injured paw
b) Favoring - the injured paw rest lightly on the floor with pressure pads not in full contact or on another part of the animal's body

c) Lifting - the injured paw elevated with the most of nails touching the floor, and the un-injured paw places firmly on the floor

d) Licking – injured paw licked or bitten

3. Still but alert

Behaviors in this category were scored when the rats put all paws firmly on the floor. The rats were allowed to rotate their heads.

4. Rest or Sleep

Behaviors in this category were scored when the rats lay down on the floor.

The Still but alert and Rest or Sleep behaviors were determined motor functions and alertness of the experimental animals.

The time spent for each of these behaviors was recorded as was summed.

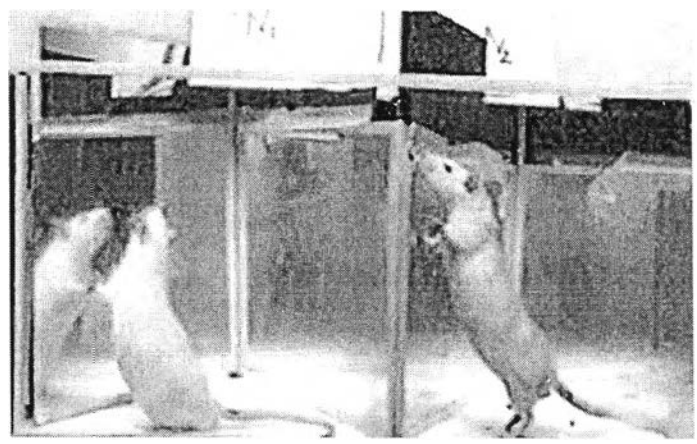

Figure [3-5] The observation chamber

Paw withdrawal test

Thermal hyperalgesia was assessed by using a radian heat source (Hargreaves et al., 1988). Each subgroup of animals was anesthetized with intraperitoneal injection of sodium pentobarbital 25 mg/kg-body-weight (Srikiatkhachorn et al., 2000). The level of anesthesia was kept comparable, based on testing of the corneal reflex. Both sides of animal paws were mark with black Indian ink before the measurements started. The nociceptive paw-withdrawal latency to radiant heat stimulation was measured using a Tail-Flick Unit (Harvard Apparatus, Kent, UK). Radiant heat originating from a 150-W infrared bulb was focused on its paws 4 cm from the distal end. Prior to thermal hyperalgesia measurement, the intensity of the heat was adjusted to 2.5 Amperes that yield a mean baseline latency of approximately 10 seconds in 10 naïve rats with cut off value, 22.5 seconds (Okamoto et. al., 2002) to avoid tissue damage.

Ten minutes after, each rat paw was placed under the radiant heat source. The period from the time when heat was applied to the paw and the time of the first paw movement (paw withdrawal latency) was measured. Ten measurements were performed in each paw. The rats' paws were allowed to wait at least one minute before next measurements were continued.

Figure [3-6] The tail flick unit

Immunohistochemical Study

Perfusion and Tissue preparation

At each of respective time point, the rats were sacrificed with over dose of sodium pentobarbital. The rats were laparotomized and were thoracotomized. They were injected with 0.5 ml of heparin into the left ventricle. Two to five minutes later, a cannula connected to a constant pressure perfusion apparatus was inserted into the apex of the heart and was advanced just distal to the aortic arch. Then, the vasculature was flushed transcardially with 250 ml phosphate buffer saline (PBS), followed by 250 ml of 4% para-formaldehyde in 0.1 M PBS pH 7.4.

After perfusion the brain was removed and was post-fixed in 4% para-formaldehyde at room temperature for 1 hour, and then that was stored in PBS at 4°C until used.

The brain-cut procedure

The sensory cortex of the thalamus of the brain was selected in this study. The brain was cut into the brain block at the bragma -2.26 mm to bragma -3.30 mm.

The brain blocks were incubated in 30% sucrose solution in PBS overnight. The brains containing thalamus were cut in axial plane in a cryostat (Microm HM 505N; Walldorf, Germany) at a thickness of 30 μm. Specimens were collected in a series of one in five sections. Sections were collected by free floating technique and were processed for immunohistochemical study of Fos protein expression.

Immunohistochemical procedure for Fos protein expressions

In immunohistochemical study, PBS with 0.1% normal horse serum (NHS) and 0.01% tritron-X 100 was used. The sections were washed by PBS three time for 10 minutes each, then they were incubated with 50% ethanol in distilled water (DW), and 3% H_2O_2 in 50% ethanol for 45 minutes, each step, respectively. After that, the sections were rinsed in three changes of PBS then, the non-specific binding of the antibody were blocked by incubating tissues with 3% normal horse serum (NHS) in PBS without tritron-X 100 for 60 minutes at room temperature. After three rinses by PBS, the sections were incubated in the anti-Fos antiserum (Santa Cruz) in 1:1000 dilutions in 3% NHS overnight (4°C).

After three times washing with PBS, the sections were incubated with Envision labelling, HRP anti-mouse anti-rabbit for 45 minutes. Then the sections were washed again by PBS three 10-minute time. After that, 33′-diaminobenzidine (DAB) was introduced to react for peroxidase activity. The reaction were stop by washing in DW at least 3 times, then the sections were placed on the glass slides then dried in room temperature overnight and were covered by cover slips.

Data Collection

1. Observed behaviors of the rats

The behaviors of the rats were classified into 4 categories as described. The data of each behavior were pooled to be the score in a thirty-minute period. Then every behavioral score of each group was calculated to the mean score of each group of treatments.

2. Paw withdrawal test

Data from each paw were averaged and were used as the latency of each rat. Then the latencies of each rat were calculated to the mean latency of each group of treatments.

3. Number of Fos positive cells

Fos protein immunoreactive (Fos-IR) neurons were distinguished by their darkly stained nucleus. A Fos-IR neurons discerned in the grid of 100 μm x 100 μm was counted. Total cells observed in ten 100 μm x 100 μm squares were counted from each slide.The counting areas are included both ipsilateral and contralateral hemispheres of somatosensory cortex for 5-8 sections/ slide/ rat.

Data Analysis

All values were presented as mean ± standard derivation (SD). The differences between groups were tested using independent sample t-test method. The differences among times series were tested using ANOVA. Significant differences between means were determined using the LSD test. Probability values of less than 0.05 were considered to be statistically significant.