

## Chapter II

### Materials and Methods

#### EXPERIMENTAL ANIMALS

Animals used in the present study are listed in the table below. All animals were male. They were housed under the condition with uniform humidity (60%), temperature ( $23 \pm 2^{\circ}\text{C}$ ) and lighting (a 12 hour light/dark cycle : on 07.00 hrs; off 19.00 hrs). All animals were allowed access to food (Gold Coin, Premium Quality Feed, KMP Feedmills Pte., Ltd.) and tap water *ad libitum*. All behavioral experiments were carried out during the day time, i.e. 9 a.m. to 5 p.m.

#### EQUIPMENT

##### A: Preparation of mitragynine

1. Rotary evaporator (Buchi, Germany)
2. Infrared spectrometer (Magna-IR Spectrometer 750, Nicolet, U.S.A.)
3. Nuclear Magnetic Resonance spectrometer (JNM-A500, JEOL, Japan)

##### B: Pharmacological Experiments

##### 1. Studies of Analgesic Activity

1. Hot Plate Analgesic Tester (Harvard Apparatus, United Kingdom)

##### 2. Locomotor Activity

1. Activity cage (Animate AT320, Sankyo, Japan)

##### 3. Body temperature

1. Digital thermometer (Delta SK-1250 ML, Sato Keiyoki Mfg, Japan)

##### 4. Rotational behavior

1. Stereotaxic apparatus with microinjection kit.(Narishige model SR-6, Japan)

Experiment	Animal	Strain	Weight (g)	No. used	Supplier
Hot plate test	Mice	Swiss albino	25-33	110	NLAC
Writhing test	Mice	Swiss albino	25-33	50	NLAC
Formalin test	Mice	Swiss albino	25-33	100	NLAC
Locomotor activity test	Mice	ddY	25-30	82	SCL Co.
Rotational behavior	Rat	Wistar	200-250	18	SCL Co.
Microdialysis	Rat	Wistar	250-300	15	NLAC
Head weaving and head twitch	Mice	Swiss albino	25-30	40	NLAC
Body temperature	Mice	ddY	25-30	24	SCL Co.
Iontophoresis	Rat	Wistar	280-350	10	NLAC

Table 1. Experimental animals used in the experiment. NLAC = National Laboratory Animal Center, Mahidol University, Nakorn Pathom. SCL Co. = SCL Co., Shizuoka, Japan.

## 5. Microdialysis

1. Stereotaxic apparatus (Narishige model SR-6, Japan)
2. HPLC system
  - Pump (ConstaMetric® 4100, Thermo Separation Products, U.S.A.)
  - C18 Reversed-phase column 250 x 4.6 mm. particle size 5  $\mu\text{m}$ ., Spherisorb ODS2 (Phenomenex® , U.S.A.)
  - guard column with packing material particle size 5  $\mu\text{m}$ ., Spherisorb ODS2
  - Electrochemical Detector (LC-4C BAS Amperometric Detector, U.S.A.)
  - Personal computer (Compaq prolinea 4/33)
3. Microinjection pump (CMA/100, Carnegie, Sweden)
4. Dialysis tube with a molecular weight cutoff point of 50,000 (Hemofilter PNF-140, Asahi Medical Co., Tokyo)

## 6. Receptor binding assay

1. Refrigerated centrifuge (Hitachi model 55P-72, Japan)
2. Liquid scintillation counter (Beckman, model LS-65)

## 7. Iontophoretic Study

1. Capillary Puller (Narishige model PY-6, Japan)
2. Electrode Puller (Narishige model PE-2, Japan)
3. Electrophysiology and microiontophoretic system
  - Amplifier (Microelectrode Amplifier model ME2-8201, Nihon Kohden, Japan)
  - Osilloscope (Dual-Beam memory osilloscope model VC-10, Nihon Kohden, Japan)

- Iontophoresis power supply (Neuro Phore BH-2 system power supply model MS-2B (Medical System Corp. U.S.A.))
- Iontophoresis pump (Neuro Phore BH-2 Iontophoresis pump model IP-2 (Medical System Corp. U.S.A.))
- Analog Digital Instruments (Maclab<sup>TM</sup>/4 AD instruments, Australia)
- Digital recorder (Macintosh® computer ,Model LC630 , Apple computer, Inc., U.S.A.) with Chart<sup>TM</sup> v.3.2.8 software for data recording system

#### CHEMICALS

1. Ammonium hydroxide (E.Merck, Germany)
2. Anhydrous sodium sulfate (Ridel de Haen, Germany)
3. Apomorphine hydrochloride (Sigma, U.S.A.)
4. Calcium chloride (Ridel de Haen, Germany)
5. Chloroform (E.Merck, Germany)
6. Citric acid (J.T. Baker, U.S.A.)
7. Chloroform (E.Merck, Germany)
8. Disodium EDTA (Sigma, U.S.A.)
9. Dihydroxyphenylacetic acid (DOPAC) (Sigma, U.S.A.)
10. Dopamine (Sigma, U.S.A.)
11. Epinephrine hydrochloride (Sigma, U.S.A.)
12. Ethyl acetate (E.Merck, Germany)
13. Formaldehyde (E.Merck, Germany)
14. Glacial acetic acid (E.Merck, Germany)
15. Haloperidol hydrochloride (Jansen Pharmaceutical)
16. n-Hexane (E.Merck, Germany)

17. Homovanillic acid (HVA) (Sigma, U.S.A.)
18. 6-Hydroxydopamine (Sigma, U.S.A.)
19. 5-Hydroxyindole-3-acetic acid (HIAA) (Sigma, U.S.A.)
20. Ketanserin (Research Biochemicals International, U.S.A.)
21. Methanol (E.Merck, Germany)
22. Methanol (HPLC grade) (E.Merck, Germany)
23. 5-methyl-N,N-dimethyltryptamine (Sigma, U.S.A.)
24. Monosodium glutamate (Sigma, U.S.A.)
25. Norepinephrine hydrochloride (NE) (Sigma, U.S.A.)
26. Pentobarbitol sodium (Nembutol<sup>®</sup>, Sanofi (France) Ltd.)
27. Petroleum ether (commercial grade)
28. Potassium chloride (Ridel de Haen, Germany)
29. Serotonine hydrochloride (5-HT) (Sigma, U.S.A.)
30. Sodium acetate (Ridel de Haen, Germany)
31. Sodium carboxy methylcellulose (Ridel de Haen, Germany)
32. Sodium chloride (Ridel de Haen, Germany)
33. Sodium octanesulfonate (Sigma, U.S.A.)
34. Tris (Sigma, U.S.A.)
35. <sup>3</sup>H-Spiperone (Amersham, Japan)
36. (-) Sulpiride (Research Biochemicals International, U.S.A.)
37. PCS II scintillation liquid (Amersham, Japan)
38. Urethane (Sigma, U.S.A.)

## EXPERIMENTAL METHODS

### A: Isolation of Mitragynine

The extraction procedure used in this study was modified from that described by Keawpradub (1990). The fresh leaves of *Mitragyna speciosa* (Korth.)

were collected from southern Thailand. The plant materials were identified by comparison with the herbarium specimen in the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

The leaves (10.2 kg) were cleaned with plenty of water and, after the excessive water was removed, let partially dried in the open air. They were then completely dried in the hot air oven at 55 °C. The dry leaves were blended into coarse powder, macerated with 95 % ethanol with periodic shaking. After 3 days of maceration, alcoholic solvent was collected and filtered. The marc was remacerated twice with another 2 successive portions of 95 % ethanol (10 liters). The combined filtrate was concentrated to yield syrupy mass under reduced pressure with a rotary evaporator. The syrupy mass was dissolved in glacial acetic acid then diluted with distilled water to give 10 % final concentration of acetic acid solution. This solution was well shaken and left standing over night then filtered. The acidic filtrate was washed with portions of petroleum ether, then made alkaline (pH 9) with 25 % ammonia solution and extracted with chloroform until complete exhaustion of alkaloids. Testing for complete extraction was carried out with Dragendoff's spray reagent. The combined chloroform extract was washed with Brine solution (saturated sodium chloride in distilled water), dried over anhydrous sodium sulfate and evaporated under reduced pressure to yield dry crude alkaloidal extract. The extraction procedure was shown diagrammatically in figure 4.

Crude alkaloidal extract (one batch of about 12 g.) was dissolved in chloroform (20 ml.) and gently placed on the top of a silica gel column (8 x 45 cm.) holding n-hexane. The column was eluted with the following of different composition and proportion (shown in parenthesis): n-hexane:chloroform (4:1), (1:1); chloroform; chloroform : methanol (97:3), (9:1), (4:1), (1:1) and then washed with methanol until no traces of alkaloid could be detected. Fractions,

each containing 50 ml, were collected and compared by TLC. The mentioned solvent systems afforded 20, 30, 30, 60, 30, 20 and 30 fractions, respectively. Those fractions of similar alkaloidal pattern and containing mitragynine, compared with the known authentic alkaloid, were combined and evaporated to dryness under reduced pressure with a rotary evaporator giving a syrupy mass.

A portion of this syrupy mass (2.5 g.) was then dissolved in chloroform (5 ml.) and gently placed on the top of silica gel column (3.5 x 45 cm.) holding n-hexane. The column was eluted with n-hexane:ethyl acetate (19:1), (9:1), and (4:1). The volumes of eluting solvents used was 500, 900 and 900 ml, respectively. Twenty ml. fractions were collected. Those fractions that contained only mitragynine when compared with the known authentic alkaloid using TLC were combined then evaporated to dry giving a yellow amorphous solid. The diagrammatic isolation method was shown in figure 5.

#### Identification of mitragynine

The yellow amorphous solid was then identified by Nuclear Magnetic Resonance, Infrared Spectroscopy and TLC. The spectra and chromatograms were compared with those of authentic mitragynine.

#### B: Pharmacological Experiments

Pharmacological studies of mitragynine were performed with the use of various experimental methods as listed below. In general, preliminary studies were carried out with the use of behavioral models. Subsequently, detailed studies were performed with specialized methods in attempt to clarify the mechanism of actions of mitragynine as indicated by behavioral studies.

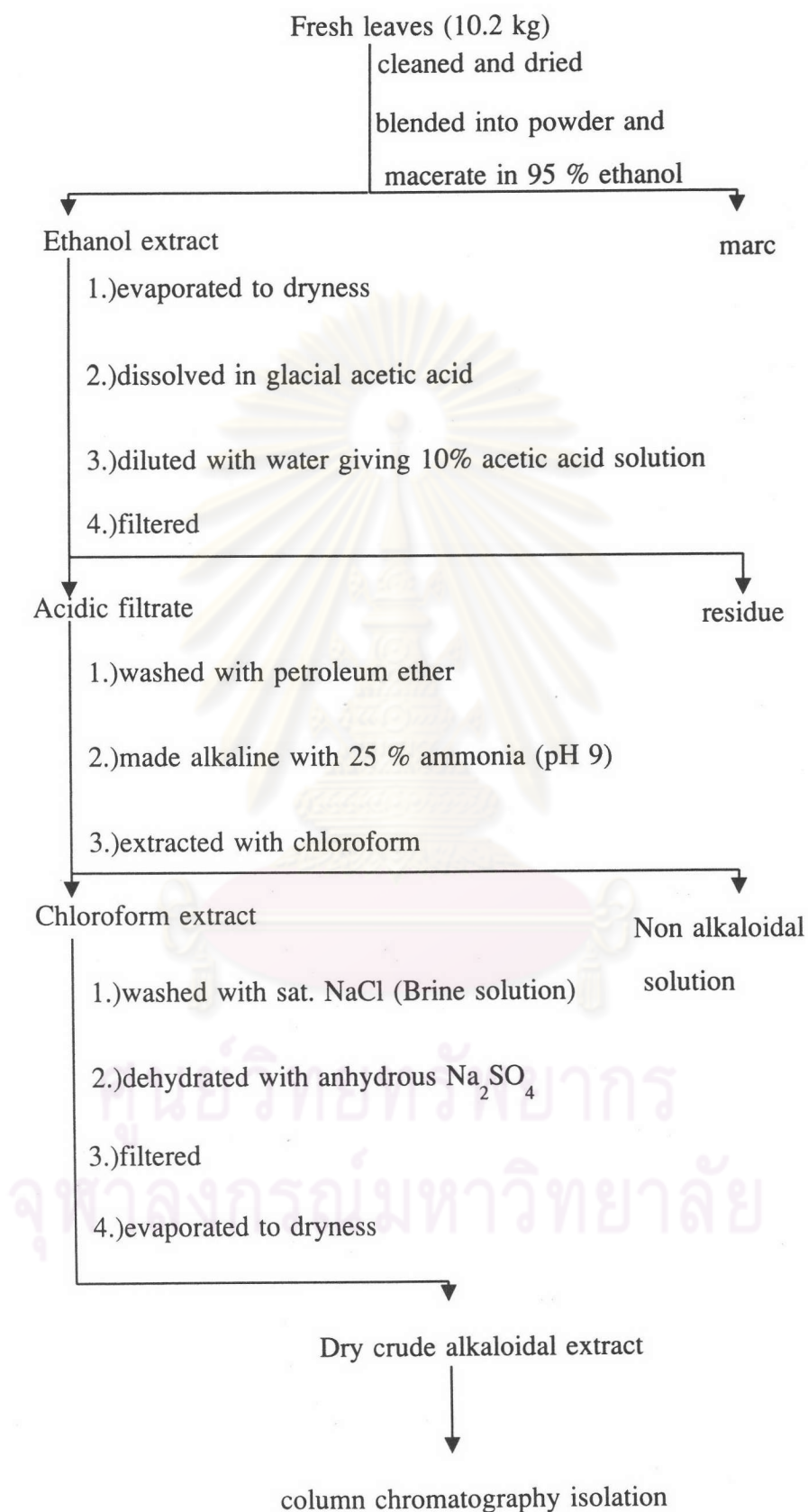


Figure 4. The extraction procedure of *Mitragyna speciosa*(Korth.)



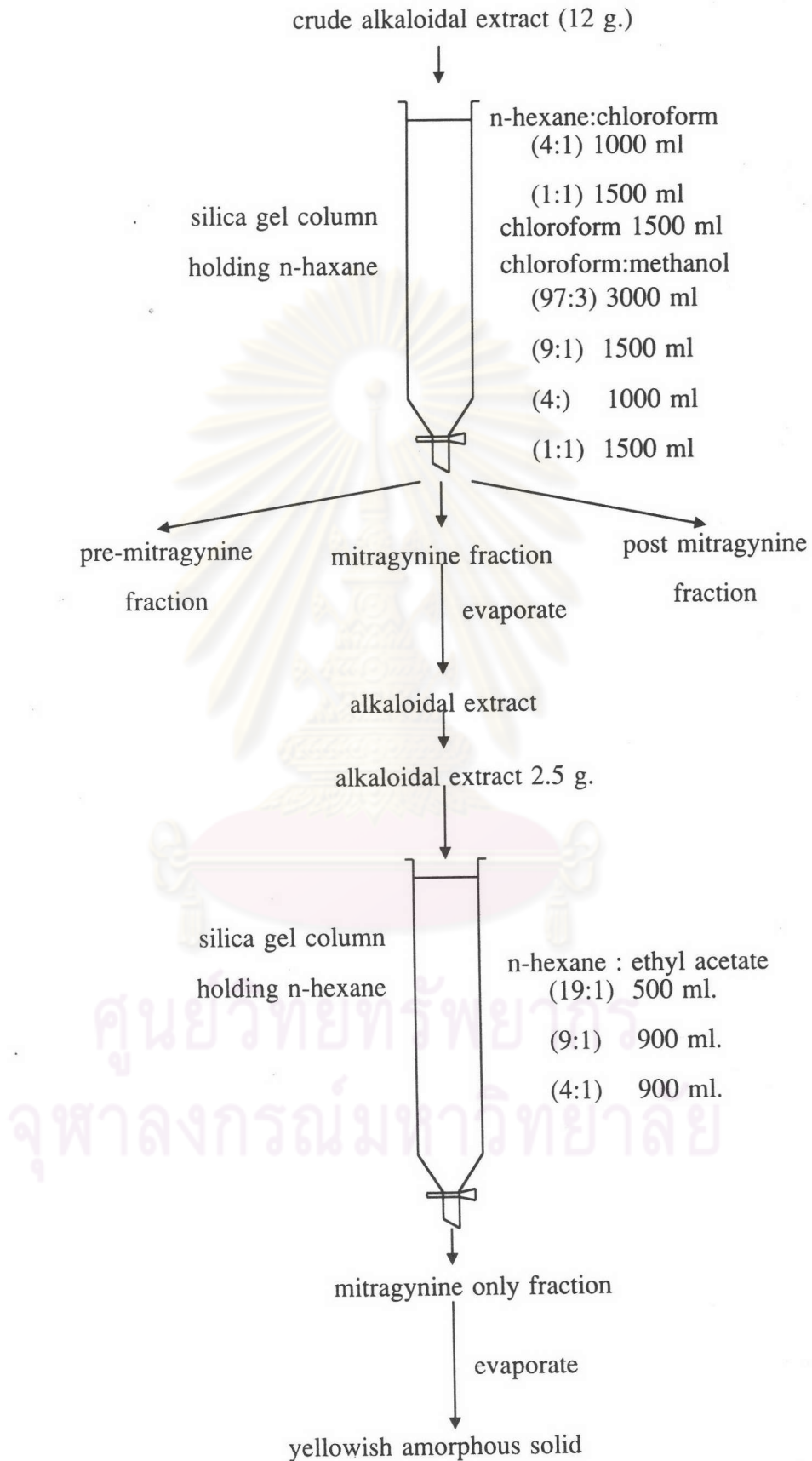


Figure 5. Isolation process of mitragynine

1. Studies of Analgesic Activity:
  - 1.1 Hot plate test
  - 1.2 Writhing test
  - 1.3 Formalin test
2. Locomotor activity
3. Body temperature
4. Microdialysis
5. Receptor binding assay
6. Head twitch and head weaving model
7. Iontophoresis study

#### 1. Studies of Analgesic Activity

##### 1.1 Hot plate test

The hot plate test was carried out according to the method described by Woolfe and MacDonal (1944) using heat as a nociceptive stimulus.

Male mice weighing 25-33 g. were used in this experiment. Mitragynine solution of different concentrations were prepared by suspending alkaloid powder in 0.5 % CMC in normal saline solution. Intraperitoneal injections were made in each group (10 mice) of animals at the doses of 15, 30, 45 and 60 mg/kg body weight respectively. Volume of each injection was 0.1 ml/10 g body weight. Control groups received 0.5 % CMC in normal saline of the same volume. Thirty minutes after injection the mice were placed on top of the hot plate ( $55 \pm 0.5$  degree Celsius). End point of the trial was determined as the duration (in second) that the mice stayed on the hot plate until they performed first paw licking or jumping in escape from the heat. The duration was recorded in mitragynine treated animals and compared with that of the control group. The cut-off time was 45 seconds, i.e. if the mice showed no response within 45 sec

latency time was assigned as 45 sec. % Maximum possible effect (%MPE) was calculated by the following equation.

$$\%MPE = \frac{[\text{jump latency}] - [\text{mean of vehicle group latency}]}{[\text{cut-off time}] - [\text{mean of vehicle group}]}$$

To determine the effect of opiate antagonist on antinociceptive effect of mitragynine, naloxone 0.1 mg/kg, morphine 10 mg/kg were injected intraperitoneally as single dose. In this study, morphine was used as positive control. Thus, another groups of mice were pretreated with naloxone 0.1 mg/kg 15 min before the intraperitoneally injection of morphine (10 mg/kg) or mitragynine (60 mg/kg). The latency of response was measured and compared as described above.

### 1.2 Writhing Test

Writhing test is a test for analgesic activity using chemical as a nociceptive stimulus (Collier et al., 1968).

Writhing test in mice was produced by i.p. injection of 0.1 ml/10 g 0.6% acetic acid. Writhing was observed 5 min after the injection by counting the number of body stretching over the period of 20 min. In experiment group (mice, male, 25-33 g), mitragynine suspension (in 0.5% CMC in normal saline) was injected 30 min prior to acetic acid injection. Mitragynine was given at the dose of 15, 30, 45, and 60 mg/kg in each group. Control group received injection of 0.5% CMC in normal saline. Each group consisted of 10 mice.

% inhibition = 100 x (1 - no. of stretching of test drug / no. of stretch of control)

### 1.3 Formalin Test

In formalin test, response to a long-lasting nociceptive stimulus was observed as a result of injecting formalin solution into the animal's paw, which produced sustained irritation. It is claimed that the pain so produced is resemblance to clinical pain (Murray et al., 1988)

Male mice weighing 25-33 g. were used in this experiment. The animals were divided into group of 10. Mitragynine suspension was prepared by suspending alkaloid powder in 0.5 % CMC in normal saline solution and was given intraperitoneally and orally at the doses of 15, 30,45 and 60 mg/kg body weight. The volume of injection was 0.1 ml/10 g body weight. 0.5 % CMC in normal saline was injected in control group. Thirty minutes after either mitragynine or control injection 20  $\mu$ l of 1% formalin solution was injected into the the paw of the right hind limb. The time duration that the mice used in licking the injected paw were recorded during each 5 min interval and continued for 45 minutes. The results recorded consecutive time intervals were compared among different group.

## 2. Locomotor Activity

Spontaneous motor activity was measured with the aid of an activity cage (Sankyo Co.) which consisted of a doughnut-shaped cage (inner and outer diameters of 16 and 32 cm respectively ; height, 13 cm) with 36 detector units which were radially arranged from the center of the cage. Each unit consisted of four pairs of photosensors containing a phototransistor( $\lambda=800$  nm) and light emitting diode (infrared with  $\lambda=950$  nm). Scanning of each detector unit (vertical scanning ; 10 times/1 ms) was controlled by one-chip microcomputer to check which pair of photosensors was detecting an animal body. A scan of all units required 36 ms (1 ms/unit x 36 units) and was controlled by a main Central

Processing Unit (Z-80), to which 36 vertical scanning data were sent and analyzed as changes in parameters of spontaneous motor activity. From scanning data, motion distance in each interval period was calculated and defined as locomotor activity. The locomotor activity of each group was compared to the control group. Diagrammatic drawing of the activity cage is shown in figure 6.

Group of 9 male ddY mice, weighing 28-32 g., were used. Two hours before each experiment, each animal was housed isolately in the cage. Counting of spontaneous locomotor started 20 minutes after each animal was placed in the doughnut-shape activity cage, the tested drugs were given to the animals 10 minutes after commencement of data collection. The data were collected at 5 minutes interval and continued for 45 min.

#### Tested Drugs

Haloperidol HCl were dissolved in normal saline solution. Mitragynine was suspended in 0.5 % CMC in normal saline solution which also used as the control. The injection volume was 0.1 ml/10 g body weight.

In single drug test, either haloperidol or mitragynine, was injected intraperitoneally 10 minutes after the experiment was started. Haloperidol was also used in pretreatment in order to observe its involvement in mitragynine induced response. In such case, haloperidol was injected i.p. 60 min prior to mitragynine injection.

### 3. Core Body Temperature

Male ddY mice weighing 28-32 g were used. Core body temperature was measured using digital thermometer, the probe of which was inserted 1.5 cm. into the rectum of the mouse. The temperature was read when it became stable. Results from different groups were compared. Mice were divide into 4

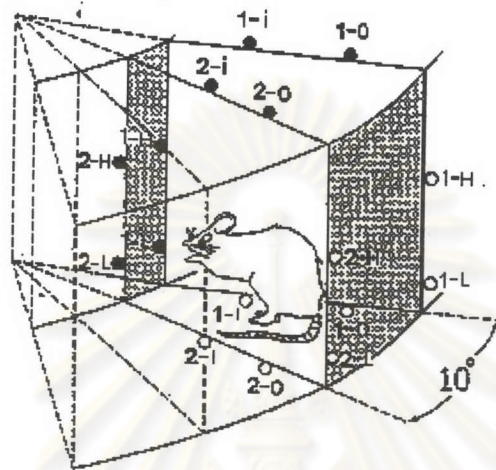


Figure 6. Schematic drawing of the system for detecting animal movement. The detection system consisted of 36 units of detectors, radially arranged from the center of the doughnut-shaped cage. H, L, O, and I represent the higher-, lower-, outer-, and inner-position sensors, respectively. Open and closed circles are phototransistors and LED, respectively. One detector unit was scanned 10 times/1 msec. Thus, it took 0.1 sec to scan all unit (1 msec/1 unit x 36 units) and send the data to the main CPU (Z-80) (Matsumoto et al., 1990)

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groups , each consisted of 6 animals. Each group received mitragynine at the dose of 15, 30 and 60 mg per kg. body weight respectively, while 0.5 % CMC in normal saline was used as control.

#### 4. Rotational Behavior (Turning Behavior)

This study was based on the method described by Ungerstedt (1971).

Male Wistar rats ,weighing 200-250 g. were used in this experiment. Under pentobarbital anesthesia (50 mg/kg, intraperitoneally), the head of the rat was positioned in a stereotaxic apparatus. Midline incision was made in the scalp and the muscle overlining the skull scraped away. Using a dental drill, a hole was made through the skull on one side (right side) over the coordinates where the substantia nigra was situated. Eight  $\mu\text{g}$  of 6- hydroxydopamine (6-OHDA ) , dissolved in 0.9% NaCl solution containing 0.025% ascorbic acid, was injected in a volume of 4  $\mu\text{l}$  into the right substantia nigra (pars reticular) through a stainless canula, 0.2 mm outer diameter, connected to a Gilmont micrometer syringe. The injection time took over 2 min. The stereotaxic coordinates of the injection site, derived from rat brain atlas (Paxinos and Watson, 1986) were A: +2.8 (from the interaural line), L:1.7, V:7.8 (from the dura). After injection was made, the scalp was sutured. The rats were left in the day light lamp to keep them warm until they became conscious. Five days after the operation, rat was injected with apomorphine, 0.1 mg/kg, to test for rotation behavior.. Rotation behavior was directly observed in cylinder shape cage, 33 cm in diameter, for 60 min after the injection of apomorphine and a 360° turn was regarded as one rotation. Testing by apomorphine continued at 5 day interval until number of turn became constant. The rats that exhibited total turn over 250 but not more than 400 turns were used in the experiment. In test group, rat was injected with mitragynine at the dose of 15 and 30 mg per kg body weight, 30

minutes before injection of apomorphine (0.1 mg/kg). Control group received 0.5% CMC in normal saline instead of mitragynine. Rotation behavior was observed and number of turns were compared with another group.

#### 5. Effect of Mitragynine on Neurotransmitter Levels; a microdialysis study.

Male Wistar rats, weighing 200-250 g, were anesthetized with pentobarbital sodium, 50 mg/kg body weight, i.p.. After complete anesthesia, the rat was fixed on a stereotaxis apparatus. The scalp was cut behind the eyes from left to right. The skull was cleared and the muscles retracted and cut. A small hole was made in each side of the skull at the position of A 0.7 mm, V 6.0 mm from the bregma according to the brain atlas (Paxinos and Watson, 1986). A stainless steel with outer diameter of 0.2 mm was inserted through these holds. One end of a dialysis tube, with a molecular weight cutoff point of 50,000 (hemofilter PNF-140, Asahi Medical Co., Tokyo), was attached to this stainless steel with epoxy resin then, after complete drying, this end was sharp cut. The dialysis tube was dragged through the brain slowly until it was properly implanted into the striatum of the animal. The outer surface of this dialysis tube was covered with Epoxy resin except the portion within the striatum region. Each end of the dialysis tube were inserted into 22 gauge stainless steel tubing. A small hole was made on the skull near the midline of the head and, as an anchor, another piece of stainless steel tubing was fixed in vertically with epoxy resin. The stainless tubings, with the dialysis tube were attached to the anchor with epoxy and allowed to dry then held with dental cement. All the holes were also sealed with dental cement. After the dental cement was set, the scalp was sutured. The rat was placed back in the cage and made warm with the day light lamp until it became conscious.



Two days after implantation, the rat was placed in a Plexiglas cage (35 x 30 x 18 cm<sup>3</sup>). One end of the dialysis tube was connected to a microdialysis pump (Carnegie Medicine model CMA/100) by a polyethylene tubing with interposition of liquid swivel and Ringer's solution (147 mM NaCl, 4.0 mM KCl and 2.3 mM CaCl<sub>2</sub>, pH 6.0) was pumped into the dialysis tube at a rate of 2  $\mu$ l per min.. After 2 hours, the effluent from the dialysis tube was collected every 20 min. into mini-tubes. An hour later mitragynine was injected intraperitoneally. The dose used for each group was 15 and 30 mg. per kg. body weight respectively. Twenty  $\mu$ l of  $5 \times 10^{-7}$  M isoproterenol solution, as an internal standard for subsequent HPLC determination, was added to each fraction of dialysate. At the completion of the experiment, the brain was excised, sliced on the cooled microtome stage and used to determine the site of the implantation of the tube. Only data from the rats with appropriately located dialysis tubes were included in the present analysis

To determine the level of DOPAC, HVA and 5-HIAA, the dialysate, mixed with an internal standard, was directly injected into the HPLC apparatus equipped with a C18 reversed phase analytic column (250 x 4.6 mm particle size 5  $\mu$ m Spherisorb ODS2) and an electrochemical detector. The apparatus was operated at a flow rate of the mobile phase controlled at 1.0 ml/min (the mobile phase composed of 100 vols. of 0.1 M of Sodium dihydrogen phosphate, 0.1 mM of disodium EDTA and 0.75 mM of sodium octanesulfonate mixed with 8 volume of acetonitrile. The pH was adjusted to 4.35 with citric acid). An amperometric control equipped with graphite electrode was used for electrochemical monitoring of the column eluent. The detector potential was maintained at 800 mV versus an Ag/AgCl reference electrode. The results are expressed as a percentage of the

pre-injection control level ( mean of the three samples collected during preinjection period).

#### 6. Dopamine D2 Receptor Binding Assay

Male Wistar rats (200-250 g; SLC Co., Shizuoka, Japan) were used in the tissue preparation. Rat was decapitated and the brain removed and dissected in the ice-cold brain template. Striatum were rapidly dissected, immersed in liquid nitrogen and kept frozen at  $-80^{\circ}\text{C}$  until use. Striata of three rats were pooled, thawed and homogenized with Teflon-glass tissue homogenizer in 5 ml of ice-cold 50 mM Tris buffer, pH 7.4. The homogenate was centrifuged at  $50,000 \times g$  (28,000 rpm Hitachi 55P-72, rotor model RP65) at  $4^{\circ}\text{C}$  for 30 minutes. The pellet obtained was washed twice more by resuspending in fresh ice-cold buffer using a handy sonicator and subsequent centrifuging under the same conditions. The final pellet was resuspended in 5 ml of the same buffer and protein content was determined using Lowry method (Lowry et al., 1951). This homogenate was subsequently diluted with buffer solution to give the final protein concentration about 0.1-0.18 mg/0.7 ml before use.

In preparation for binding to D2 receptors with  $^3\text{H}$ -Spiperone (Amersham Japan, 21.3 Ci 2 mM), each tube contained 100  $\mu\text{l}$  of test agents in 50 mM Tris buffer (pH 7.4) 100  $\mu\text{l}$  of  $^3\text{H}$ -Spiperone (final concentration, 0.25 nM), 100  $\mu\text{l}$  of 1 M of Ketanserin (to block the binding to serotonin receptor) and 700  $\mu\text{l}$  of striatum tissue preparation. To determine non-specific binding ("blank condition") and total binding, (-) sulpiride at the final concentration of 10  $\mu\text{M}$  and 50 mM Tris buffer (pH 7.4) was used instead of the test agents respectively. Tubes were incubated in a shaking water bath for 90 min at  $20^{\circ}\text{C}$ . Samples were filtered through the filter sheet (Whatman GF/B) under vacuum, which were washed twice with 5 ml of ice-cold Tris buffer (pH 7.7) punched into 3 cm circled

and counted for tritium in about 8 ml PCS II scintillation liquid (Amersham) using a liquid scintillater counter(Beckman model LS-65).

#### 7. Head Twitch and Head Weaving

Head twitch and head weaving model is employed for testing serotonergic activity of tested drug.

Male mice weighing 25-33 g. were used in this experiment. Mitragynine solution was prepared by suspending the alkaloid powder in 0.5 % CMC in normal saline solution and was injected in the dose of 10, 20 and 30 mg/kg body weight. 0.5 % CMC in normal saline was injected in control group. Head weaving and head twitch were observed for 10 minutes after injection. 30 minutes after injection the mice were injected with 5-methyl-N,N,-dimethyltryptamine (5-MeO-DMT, a serotonin agonist). Head weaving and head twitch were also observed for 10 minutes after injection. Number of head weaving and head twitch were compared among different groups.

#### 8. Effect on Single Neuron: a microiontophoretic study

To study the effect of mitragynine on neuronal cell, the technique employed was microiontophoresis.

#### Preparation of borosilicate glass capillary

Borosilicate glass capillary was prepared from borosilicate tube, 10 mm. outer diameter and 8 mm. inner diameter, using capillary puller to produce capillary tubing with outer diameter of 0.2 - 0.3 mm. This capillary was inserted into another borosilicate tube and was fixed to the tube by epoxy resin. The tube with capillary tubing inside was then pulled giving a capillary tube with outer diameter of  $1.55 \pm 0.05$  mm resulting in the capillary with a small glass tubing

inside, so called omega dotted tubing. This small glass tubing was used to produce a capillary attraction to facilitate filling of the solution into the tip of the electrode to be produced subsequently . These capillaries were cut into pieces at the length of 7.5 and 10 cm and cleaned by immersion in cleansing solution for 12 hours. The capillaries were then washed by running tap water for several hours to ensure complete removal of the cleansing solution. Finally, they were rinsed with distilled water , dried within the hot air oven and kept in closed container .

#### Preparation of seven-barrel microelectrode

Seven-barrel microelectrode was prepared from a 7-barrel assembly made from glass tubings described above. The assembly was prepared by putting seven capillaries together with one capillary in the center and six others forming peripheral barrels. This assembly was fastened with a heat shrink tubing. The center capillaries was made longer than the others in order to be held easily by the electrode holder . At this end, in order to prevent the contamination and electrical coupling, the outer capillaries were melted and slightly bent away from the center barrel. All the capillaries were fixed together with 5-minute setting epoxy resin and after the epoxy resin was completely set, it was pulled to make a microelectrode using vertical type electrode puller (Narishige, model PE-2). Method of pulling comprised 2 steps. First, the set of capillaries was melt and made 2/3 turn . Finally, the capillaries were pulled down initially under gravity and subsequently by magnetic force to yield fine pointed seven barrel electrode with submicron tip diameter. The tip of the electrode was broken back by the gentle collision of a stainless steel rod under microscopic control with the assistance of micromanipulator to produce the tip with the outer diameter between 4-8 micron.

Filling the solutions.

The drug solutions used in this experiment were

1. Glutamate solution.

Monosodium glutamate was dissolved in distilled water , pH adjusted to 7 with 0.1 M HCl yielding concentration of 0.2 M.

2. Norepinephrine solution.

Norepinephrine HCl was dissolved in distilled water , pH adjusted to 5 with 0.1 M HCl or 0.1 M NaOH yielding concentration of 0.2 M.

3. Mitragynine solution.

Mitragynine was dissolved by adding small amount of glacial acetic acid and then diluted with distilled water, pH adjusted to 4 with glacial acetic acid or 0.1 M NaOH, providing 0.2 M solution.

4. Serotonin (5-hydroxytryptamine, 5-HT) solution.

Serotonin HCl was dissolved in distilled water , pH adjusted to 4 with 0.1 M HCl or 0.1 M NaOH yielding concentration of 0.2 M.

5. Gamma amino butyric acid (GABA) solution.

Gamma amino butyric acid was dissolved in distilled water , pH adjusted to 3.5 with 0.1 M HCl or 0.1 M NaOH yielding concentration of 0.2 M.

6. 4 M sodium chloride.

Sodium chloride was dissolved in distilled water giving 4 M sodium chloride solution used in filling the recording barrel, usually the center barrel.

7. 2 M sodium chloride.

Sodium chloride was dissolved in distilled water giving 2 M sodium chloride solution used in filling the current balancing barrel.

Each solution was filled in each individual barrel respectively by slowly injection through a 27 G needle that was inserted into deepest the capillary to avoid air bubbles formation.

#### Preparation of the animal

Male Wistar rat, weighing 200-250 g, were anesthetized with urethane 1.8 g/kg ,i.p.. After complete anesthesia, the head was shaved and fixed on a stereotaxis apparatus. Mid line incision was made in the scalp in anterior-posterior direction and the skull over the occipital quadrant was removed to expose the cerebellum. The dura matter was removed carefully, and the pia covering the vermis cut. Microelectrode, with the aid of micromanipulator, was inserted through the pia slit into cerebellum to record spontaneous firing of the Purkinje cell. After the cell was captured, the substance contained in each electrode barrel was released into the brain tissue using microiontophoretic injection. The change in firing rate of Purkinje cell as the result of the injection was detected and analyzed.

#### STATISTIC ANALYSIS

Data presented are expressed as mean  $\pm$  standard error of the mean (S.E.M.). Statistic analyses of changes were performed using unpaired t-test at  $p < 0.05$