CHAPTER III

MATERIALS AND METHODS

A. Animals

Adult male Wistar rats weighing between 200-210 g (approximately 7 weeks of age) at the beginning of the experiments were obtained from the National Laboratory Animal Center, Mahidol Univerity (NLAC-MU), Thailand. All animals were housed in two per cage and maintained at 25±2 °C on 12 h light/dark cycles with lights on at 06:00 a.m. Standard rat chow and water were given *ad libitum*, except during the application of restraint stress. All procedures in this study were approved by the Animal Use Committee, Faculty of Veterinary Science, Chulalongkorn University.

B. Chemicals

Behavioral test

Naloxone hydrochloride (NX), D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP, μ-opioid receptor antagonist), natridole hydrochloride (NT, δ-opioid receptor antagonist) and nor-binaltorphimine dihydrochloride (nor-BNI; κ-opioid receptor antagonist) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

The high-performance liquid chromatography (HPLC) analysis

Standard dopamine (DA), 3-4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3,4-dihydroxy-benzyl-amine hydrobromide (DHBA) and perchloric acid were purchased from Sigma Chemical Co., except methanol, heptane sulfonate, sodium dihydrogen phosphate, and Na_2·EDTA which were purchased from BDH Chemical Ltd. (London, England).

Protein measurement

Sodium carbonate (Na_2CO_3), Sodium tartrate, Copper sulphate pentahydrate ($CuSo_4 \times 5H_2O$) and folin-cioculteau phenol were purchased from Sigma Chemical Co.

Corticosterone measurement

Enzyme linked immunoassay kit (ELISA kit) for rat corticosterone was purchased from R&D system Inc. (Menneapolis, MN, U.S.A.).

C. Experimental protocols

In order to test hypotheses, this study was divided into 2 experiments as follows:

Experiment 1: To determine the effects of chronic restraint stress on sweet food intake and dopaminergic neurotransmission.

Experiment 2: To examine type of opioid receptors mediated chronic restraint stress-induced sweet food intake.

Experiment 1: To determine the effects of chronic restraint stress on sweet food intake and dopaminergic neurotransmission

Male Wistar rats were divided into 4 groups;

1. Control (CON)
2. Stress (ST)
3. Control with sweet food (CON+FL)
4. Stress with sweet food (ST+FL)

After 5 day adaptation period, the rats in stress and ST+FL group were underwent restraint stress procedure. After the end of stress session, CON+FL and ST+FL rats were trained to eat sweet food for 3 days and followed with test for sweet food preferences on the day after behavioral training (Fig. 3-1). At the end of behavioral studies, rats were immediately sedated with isoflurane (Terrell™; Minrad Inc.,Bethlehem, PA, USA). Animals were then killed by removing whole blood from heart. Whole brains were quickly removed and frozen in aluminum foil (-70° C in liquid nitrogen) for measurement of DA, DOPAC and HVA levels by HPLC-EC technique in frontal cortex, amygdala, hippocampus and nucleus accumbens. The adrenal gland weight, whole

blood and serum were also collected for further corticosterone and complete blood count analysis as an indirect parameter of hypothalamo-pituitary-adrenal axis activation.

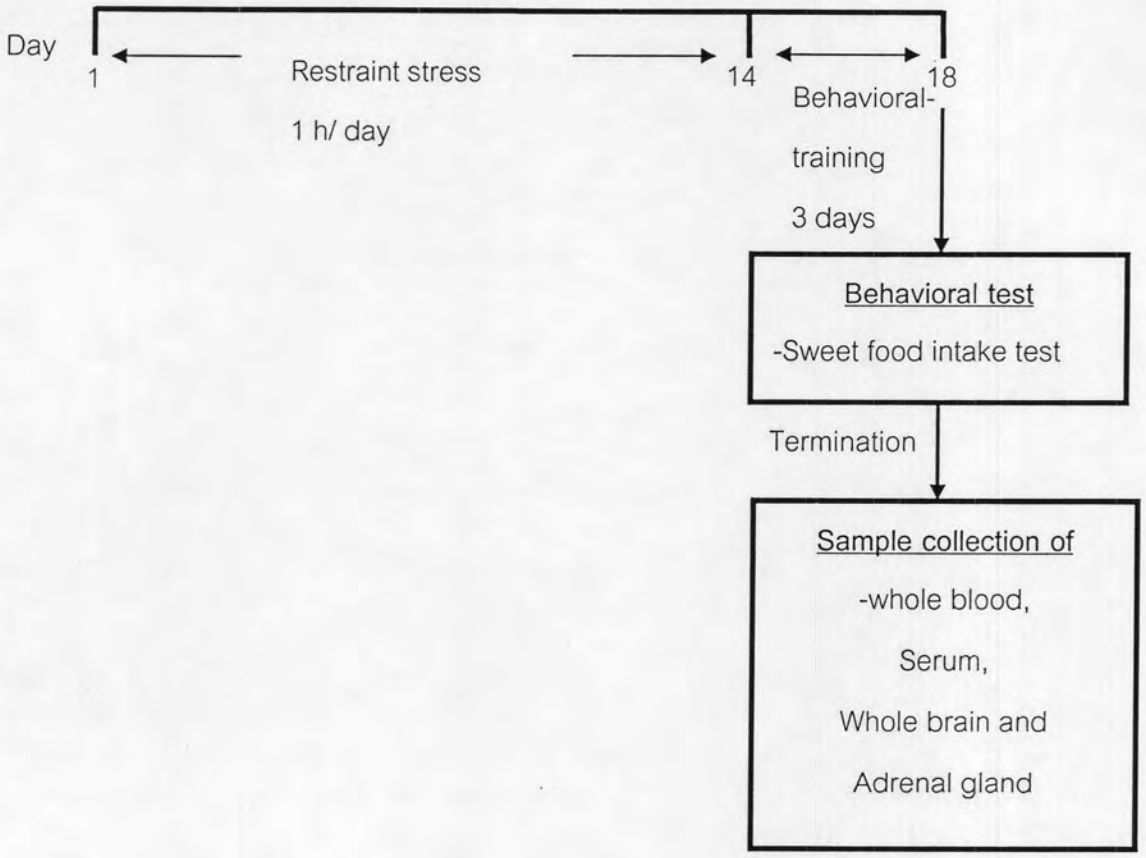


Figure 3-1 Diagram of the experimental protocol

Experiment 2: To examine types of opioid receptor mediated chronic restraint stress induced sweet food intake.

Rats were divided into 2 main groups and were divided in to 4 subgroups, as follows;

1. Control +FL

1.1 Control + FL+ vehicle

1.2. Control + FL + CTOP (μ-receptor antagonist)

1.3. Control + FL+ Naltridole (δ-receptor antagonist)

1.4. Control + FL + Nor-BNI (κ-receptor antagonist)

2. Stress + FL

2.1. Stress + FL + vehicle

2.2. Stress + FL + CTOP (μ -receptor antagonist)

2.3 Stress + FL + Naltridole (δ -receptor antagonist)

2.4 Stress + FL+ Nor-BNI (κ -receptor antagonist)

The protocol was similar to the first experiment but some rats were subcutaneously injected with selective opioid antagonist or equivalent volume of vehicle 30 min before sweet food intake test. After behavioral test, the locomotor activity was measured with open field test in order to clarify whether the motor activity was affected by antagonist leading to alter chronic stress-induced sweet food intake.

D. Methods

1. Restraint stress

Restraint stress was applied to all rats in ST and ST+FL groups by placing the animal inside a 25 × 5-cm plastic bottle, and fixing the bottle with adhesive tape on the outside so that the animals were unable to move. However, the restrainer has 1 cm hole in the far end for breathing. Instead of submitting to restrainer, all rats in control groups were gently handled every day, duration the period of restraint stress. The restraint procedure was 1 h/day performing between 10:00 and 11:00 a.m. for 14 days. The restraint stress model was done according to the method described by Ely et al., 1997.

2. Measurement of body weight, food intake and adrenal gland weight

Body weights (BW) and food intake of the animal were measured daily to determine the physiological changes. They were calculated and represented as the average daily weight gain (ADG) or the percent change of body weight (%change of BW) and the daily food intake (DFI) as the followed equation;

$$\text{ADG (g/d)} = \frac{\text{Final BW (g) - Initial BW (g)}}{\text{Day of experiment (days)}}$$

$$\% \text{ change of BW} = \frac{\text{(Final BW (g) - Initial BW (g)}}{\text{Initial BW (g)}} \times 100$$

$$\text{DFI (g/rat/d)} = \frac{\text{Sum of daily food intake (g)}}{\text{Day of experiment (days)}}$$

Adrenal gland weight which is the classical indicator of stress via hypothalamo-pituitary-adrenal axis activation was determined immediately after sacrificed. To normalize adrenal gland size to the body weight variation among the animals, the adrenal gland weight index was calculated percent of adrenal gland weight to body weight as followed;

$$\text{Adrenal gland mass index (\%)} = \frac{\text{AGW (g)}}{\text{Final BW (g)}} \times 100$$

3. The measurement of corticosterone

Blood sample for hormone assays were collected by cardiac puncture before sacrificed and were transferred to Eppendorf tubes and centrifuged at 5000 g for 30 min. Serum were collected and frozen at -20°c until they were analyzed. Corticosterone concentrations were measured by EIA kit according to manufacturer's instructions. All data for corticosterone represent the averages of each sample done in duplicate. Corticosterone concentrations were expressed as nanogram per milliliter.

4. Hematology

Blood sample was collected from cardiac puncture before sacrificed. Blood sample for hematology was transferred into EDTA tubes in order to inhibit blood clot. Total numbers of red blood cells (RBC), white blood cells (WBC) and differential counted numbered of white blood cells were automatically counted by automated machine (Coulter T890, Diamond Diagnostics Inc., Holliston, MA, U.S.A.).

5. Behavioral assessment

5.1 Sweet feeding behavior test

The procedure of sweet food preference was adopted from previous study of Silveira and co-workers (2000). Briefly, the animals were placed in a rectangular box (40x15x20 cm). Ten Froot loops®, Kellogg's pellets of wheat and corn

starch and sucrose (Nhong Shim Kellogg Co. LTD., Anseong-Si, Gyenggi-Do, South Korea) were placed at one corner of the box. Each animal was submitted to 3 days of habituation trials lasting 6 min each in order to become familiar with this food. On last day (the 3rd day) of sweet food training, if the animal ate less than 1 pellet, they would be excluded from this study. For the sweet food intake index, the numbers of ingested froot loops pellets were measured after the animals were exposed for 6 min for the test session performed on the next day of sweet food training. Fraction numbers of froot loops® they ate were determined as part of a quarter of whole froot loops® (e.g. 1/3, 1/4).

5.2 Open field test

After the sweet feeding session, only the animals of experiment 2 were tested in the open field (76 cm long x 57 cm wide x 35 cm high; Figure 3-2) for 5 min to measure locomotor activity. The open field test was used in accordance to the methods described by McCarthy et al. (1995). The numbers of total crosses that the rat made during the 5 min in this task were recorded as the locomotor activity. The experiments were recorded by a video camera for later analyzed.

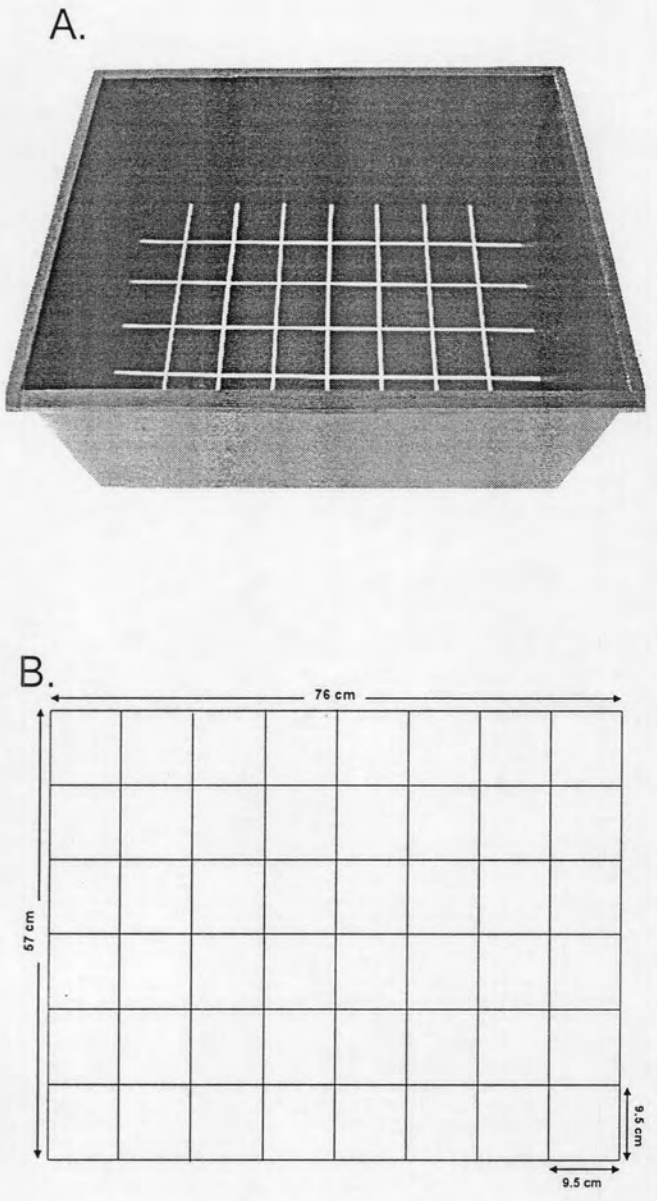


Figure 3-2 (A) Photograph of open field apparatus, a wooden box (76 cm long × 57 cm wide × 35 cm high) with (B) a floor of 48-square grid floor (6 × 8 squares, 9.5 cm per side).

6. Pharmacological treatment

For the non-selective and selective μ-, δ- or κ- opioid receptor antagonists treated group, some rats were respectively injected with naloxone, CTOP, natridole or nor-BNI at a dosage of 0.5 or 1 mg/kg BW, subcutaneously 30 min before sweet food intake test. In the vehicle group, rats were subcutaneously injected with an equivalent volume of ethanol at the dose of 1 ml/kg BW.

7. Brain dissection

After the open field test, the rats were euthanized and their brains were rapidly removed, frozen in liquid nitrogen, and stored at -70 °C. Brains were isolated into each area (followed the instruction of Heffner et al., 1980) and placed in 1.5 ml Eppendorf tubes. Briefly, a frozen brain was placed on its dorsal surface in the trough of the brain cutting block (Figure 3-3). All of the following procedures were done on ice. Razor blades were carefully inserted through the cutting channels slicing the brain at right angles to the sagittal axis. This initial razor blade slices through the coronal plane of the brain at the level of the anterior commissure. The position of the initial razor blade served as a reference point from which brain sections were obtained. Total of eight razor blades were inserted anterior or posterior to the first blade as shown in Figure 3-3.

The brain was thus divided into 8 sections (Figure 3-4). The razor blades were removed from the block with coronal brain slices adhering to their surfaces and were placed on a glass plate suspended on ice. Brain regions were then bilaterally dissected from these slices. The frontal cortex, nucleus accumbens, amygdala and hippocampus were used for HPLC.

The frontal cortex was consisted of the frontal poles, cortical tissue from section 1 (see Figure 3-4), as well as the cortical tissue superior to the rhinal sulcus from sections 2 and 3 (Figure 3-4). The nucleus accumbens was dissected from the rostral surface of the third brain section (Figure 3-4). The amygdala included the tissue lateral to both portions of the hypothalamus (sections 5 and 6) and ventral to the rhinal sulcus. The hippocampus was separated from the midbrain and overlying cerebral cortex from sections 7 and 8 (Figures 3-4) based on its distinct morphological appearance.

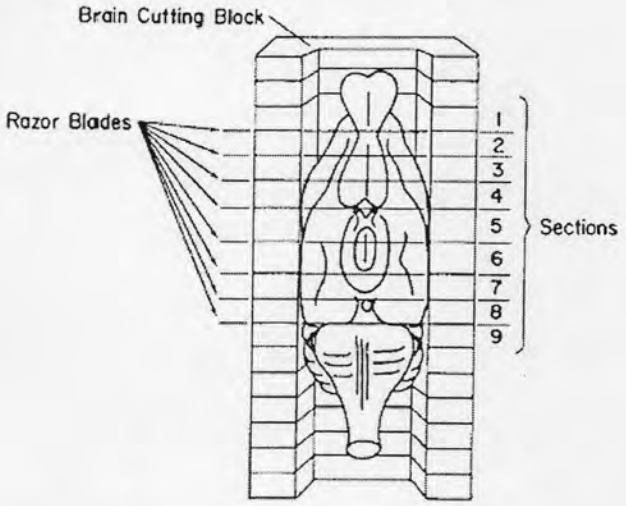


Figure 3-3 Diagrammatic representation of brain cutting block illustrating orientation of brain and placement of razor blades to obtain coronal brain sections. The numbers on the right refer to brain sections (Heffner et al., 1980).

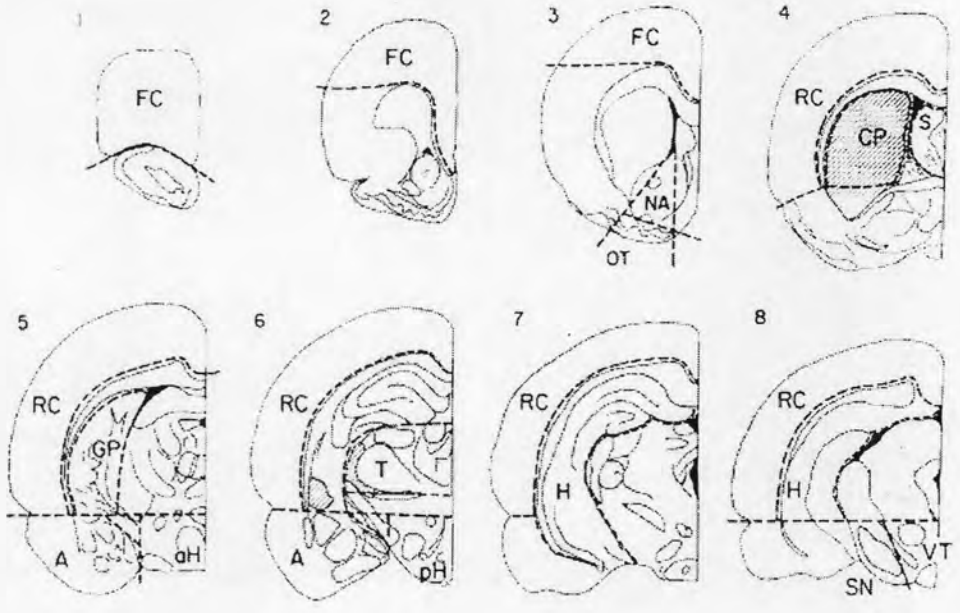


Figure 3-4 Diagrammatic representation of coronal brain sections from which brain regions are dissected. Dotted lines indicate borders of brain regions. FC, frontal cortex; NA, nucleus accumbens; OT, olfactory tubercle; S, septum; CP, caudate putamen; RC, remaining cortex; GP, globus pallidus; aH, anterior hypothalamus; pH, posterior hypothalamus; A, amygdala; T, thalamus; SN, substantia nigra; VT, ventral tegmentum; H, hippocampus. Numbers correspond to brain sections shown in Figure 3-3 (Heffner et al., 1980).

8. Neurochemical analysis

The isolated brains were sonicated in the cold 0.1 M perchloric acid containing 3,4-dihydroxy-benzyl-amine hydrobromide (DHBA), as an internal standard. Before the centrifugation, 20 µl of brain homogenate was collected for protein determination. After that samples were centrifuged at 5,000 x g (Universal 32/32R, Hettich zentrifugen GmbH & Co.KG, Germany) for 30 min at 4°C. The supernatants were collected and stored at -20°C for further analysis of monoamine neurotransmitters using HPLC with electrochemical detector (HPLC-EC).

8.1 HPLC-analysis

HPLC-EC, a glassy carbon working electrode and amperometric control (Bioanalytical systems, West Lafayette, IN, U.S.A.) were used to quantify neurotransmitter levels. A Shimadzu Model LC-10 AD pump (Kyoto, Japan) was connected to a Rheodyne injector (Cotati, CA, U.S.A.), equipped with a 20 µl fixed loop and a 15-cm phenomenex® column (Phenomenex, USA), packed with 5-µm particles. The mobile phase solution was composed of 1 mM Heptane sulfonate, 100 mM Sodium dihydrogen phosphate, 1 mM Na_2·EDTA and 5% Methanol, adjusted to pH 4.1 with saturated citric acid. The mobile phase was filtered through a 0.22-µm filter, degassed by ultrasonic agitation and pumped at a flow-rate of 1.0 ml/min. The amperometer was set at a positive potential of 0.700 V with respect to the Ag/AgCl reference electrode, with a sensitivity of 2 nAmp. The supernatant (40 µl) from the brain was injected into the HPLC-EC system to detect the DA and its metabolites DOPAC and HVA.

Delta 5.0 software (Digital Solutions, Margate, QLD, Australia) was used to analyze the chromatography data.

8.2 Analytical procedures

Standard solutions at different concentrations were injected into HPLC system. The retention time was evaluated by injecting the standard dopamine and its metabolites individually and by the injection of a standard mixture (Figure 3-5). The concentrations of transmitters and metabolites were calculated by reference to standards and internal standard using peak integration and expressed as pmol/mg

protein of brain tissue. Protein concentration of various brain regions were determined by the method of Lowry et al. (1951).

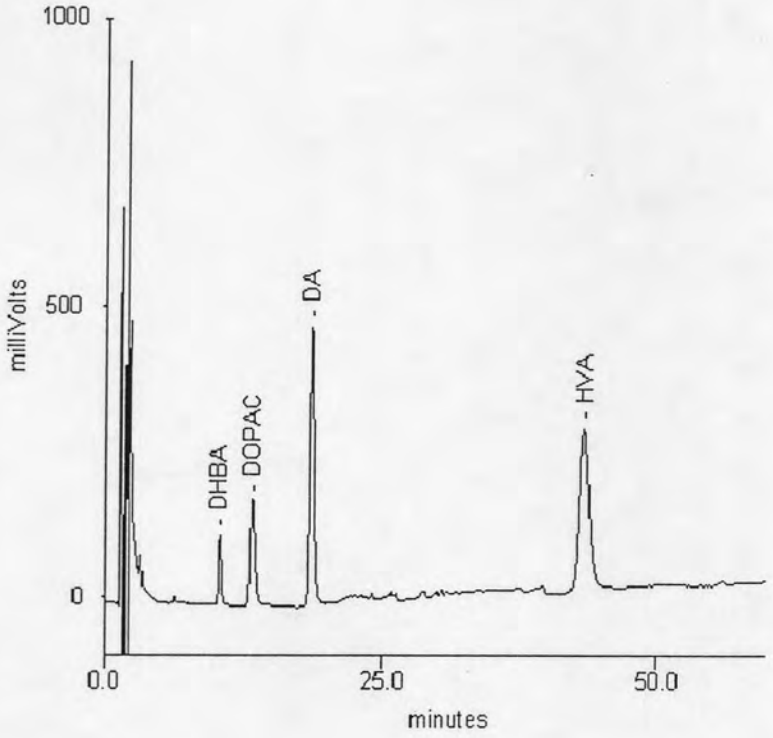


Figure 3-5 The chromatogram represents peaks of standard DHBA, DOPAC, DA and HVA measured by HPLC-EC. The retention times of DOPAC, DA and HVA were approximately 10, 13.29, 18.17 and 42.26 minutes, respectively.

9. Measurement of protein concentration

Total protein concentrations of each brain homogenate were measured according to the Lowry's method (Lowry et al., 1951).

9.1 Reagents

Reagent A was 2% Na_2CO_3 in 0.1 M NaOH. Reagent B was 4% sodium tartrate. Reagent C was 2% $CuSO_4 \cdot 5H_2O$. Reagent D was freshly prepared from mixing of reagent A, B and C at a ratio of 200:1:1. Folin-cioculteau phenol reagent was diluted 1:2 in H_2O before use. Bovine serum albumin (BSA) was used as a standard.

9.2 Procedure

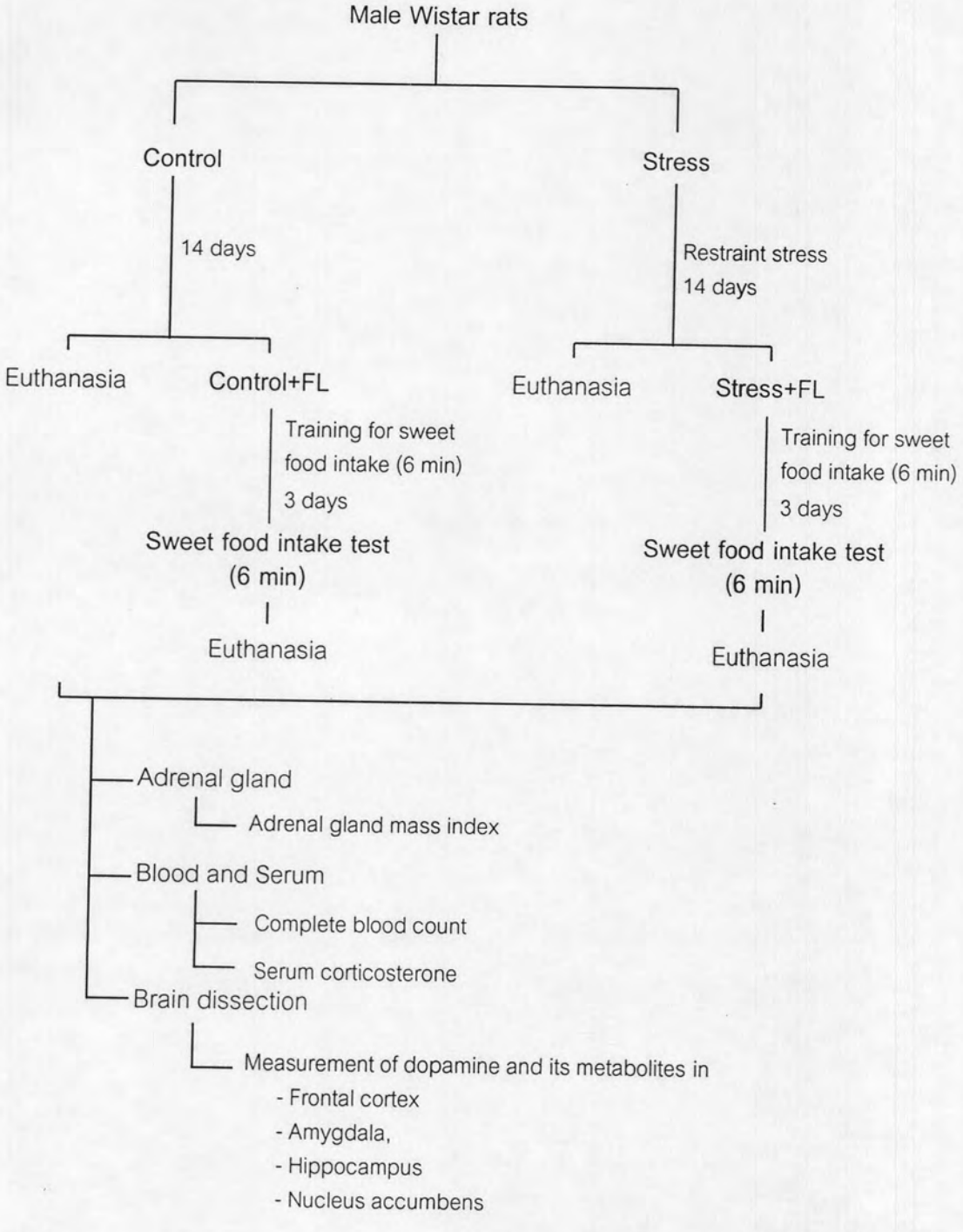
Sample or standard at a volume of 100 µl was added with reagent A (100 µl) and followed by 1 ml of freshly reagent D. Diluted Folin reagent 100 µl was rapidly added to the mixture and allowed to interact for 30 min at room temperature. The absorbance was then measured at a wavelength of 750 nm. A standard absorbance curve of known concentration of standard BSA (0-1 mg/ml) was plotted as a linear correlation for determination of the unknown protein concentration.

10. Statistical analysis

All data were presented as means ± standard errors of the mean (SEM). In the first experiment, two way analysis of variance (two-way ANOVA) was used to compare the effect of stress or sweet food on each parameter, and followed by Student Newman Keuls as appropriate. If the two-way ANOVA revealed the interaction between 2 effects (i.e. stress x sweet food), the one-way ANOVA followed by Student Newman Keuls was used to compare between groups. The sweet food ingestion was analyzed by unpaired *t*-test to compare between control+FL and stress+FL groups. In the second experiment, the sweet food intake index, the levels of neurotransmitters and the locomotor activity were analyzed by one-way ANOVA and followed by Dunnett's *t*-test to compare between vehicle and the effect of opioid antagonists. Differences were considered statistically significant at $P<0.05$.

E. Summary of experimental design

Experiment 1: To examine the effects of chronic restraint stress on sweet food intake and dopaminergic neurotransmission



FL= Froot loops®

Experiment 2: To examine type of opioid receptors mediates chronic restraint stress induced sweet food intake.