CHAPTER IV
MATERIALS AND METHODS

ANIMALS

Wistar rats were supplied by the National Laboratory Animal Center of Mahidol University Salaya Campus.

CHEMICALS

1. Animal Preparations

Pento barbiturate sodium (Nembutal®) was purchased from Sanofi (Thailand) Ltd. Normal saline was purchased from Hospital Products Public Co, Ltd. Glyceryl trinitrate (NTG) was purchased from DBL Thailand. Parachlorophenylalanine (PCPA) was purchased from Research Biochemical International (RBI), USA.

2. Intravital Microscopic Study

Fluorescein isothiocyanate-dextran (MW 150 D) (FITC) was purchased from Sigma, USA. Sodium chloride (NaCl) and sodium carbonate ($NaHCO_3$) was purchased from Merck. Temp-Bond was purchased from Kerr Corporation. Calcium chloride ($CaCl_2$), potassium chloride (KCl) and magnesium sulphate ($MgSO_4$) were purchased from Riedel-de Hach, Germany.

3. Electron Microscopic Study

Ethanol was purchased from Merck. Propylene oxide was purchased from Serva. Permount was purchased from Fisher Scientific USA. Epon 812, glutaraldehyde, lead citrate, osmium tetroxide, toluidine blue and uranyl acetate were purchased from Electron Microscopic Sciences, USA.

4. Immunohistochemical Study

Chemicals reagents: Tissue freezing medium was purchased from Jung, Germany. Bovine serum albumin (BSA), 3,3-diaminobenzidine (DAB), hydrochloric acid (HCl), paraformaldehyde, potassium phosphate (KH_2PO_4) and sucrose were purchased from Sigma, USA. Hydrogen peroxide (H_2O_2), sodium chloride (NaCl), di-sodium hydrogen phosphate (Na_2HPO_4), sodium hydroxide (NaOH), tris (hydroxymethyl)-aminomethane and triton X-100 were purchased from Merck, USA. Gelatin was purchased from Boehringer Ingelheim Bioproducts Partnership. ABC streptavidin-horseradish peroxidase complex was purchased from Vector.

Antibodies: Sheep antibody polyclonal against c-fos, synthetic c-fos peptide (residues Ser, Gly, Phe, Asn, Ala, Asp, Tyr, Glu, Ala, Ser, Ser, Arg, Cys) conjugated to bovine thyroglobulin (Code No. AB 1548; Lot No. 17120013), biotinylated rabbit antisheep IgG (Code No. AP 147B; Lot No. 17068030) and normal rabbit serum (Code No. S-20; Lot No. 17090246) were purchased from Chemicon International, USA.

METHODS

EXPERIMENTAL ANIMALS

Adult male Wistar Furth rats weighing 300-350 grams were used in this study. The animals were housed five per cage in stainless-steel bottom cages. They were kept in a well-ventilated room in which the temperature was 28-32 ^{0}C with an automatic lighting schedule which provided darkness from 7.00 PM to 6.00 AM. All animals were allowed access of food (Purina Laboratory Chow, Premium quality feed, Zuelig Gold Coin Mills Pte., Ltd., Singapore) and tap water ad libitum. To limit the effects of nonspecific stress, all animals were accustomed to daily handing for at least 5 days before experimentation.

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

1. Controlled group: The experimental rats were injected with physiological normal saline of the same volume as experimental group, intraperitoneally.

2. Experimental group: The experimental rats were injected with parachlorophenylalanine (PCPA), a tryptophan hydroxylase inhibitor, 300 mg/kg, intraperitoneally.

INDUCTION OF HYPOSEROTONIN STATE

The serotonin depletion agent used in the present study was para-chlorophenylalanine (PCPA). PCPA is an enzyme inhibitor most widely used in experiments. It inhibits tryptophan hydroxylase irreversibly to cause a long-lasting reduction in 5-HT level. In this study, the animals were chosen by randomly and were injected by PCPA (300 mg/kg, intraperitoneally). Control animals received an equal volume of normal

saline. Three days after induction, the animals were then chosen for the further experiments (Sjoerdsma et al., 1970).

SURGICAL PREPARATION

Experimental rats were anesthetized for the duration of experiments by intraperitoneal administration of 50 mg/kg of sodium pentobarbital. Additional doses of anesthetics were given as required to maintain surgical anesthesia based on testing of corneal reflex and response to tail pinch. After tracheostomy, the ventilation was assured by using positive pressure ventilator. (rodent ventilator model 683, Harvard Apparatus, USA) A femoral artery and vein were cannulated to record blood pressure and for intravenously infusion of drugs, respectively. Blood pressure was monitored throughout experiments with pressure transducer (Nikon model TP-300T) which recorded on polygraph (Nikon RM 6000, Nikon Khoden, Japan). Arterial blood was collected periodically for determination of pH, PaO_2 and $PaCO_2$ by the pH/blood gas analyzer (238 pH/blood gas analyzer, Ciba Corning Diagnostics, UK). The procedures of animal preparation were concluded in diagram as shown in Figure 4.1.

INTRAVITAL FLUORESCEIN VIDEOMONITORING TECHNIQUE

After tracheostomy and cannulation had been performed, the rat was placed on a surgical frame and the head was securely positioned with a head holder. The skull was exposed and the frontal bone was thinned by drilling with saline-cooled drill, until the blood vessels of dura became clearly visible. The superior sagittal sinus was exposed and the dura was then opened using a microneedle. A cranial window was placed over craniotomy opening. An artificial cerebrospinal fluid was infused into the intracranial space. After placing cranial window, the animal was

placed under the fluorescein microscope (Optiphot 2, Nikon, Japan) fitted with the videomonitoring system (SIT Camera, DAGE). Fluorescein-isothiocyanate labeled dextran (FITC-Dx-150) (MW 150,000), a fluorescent plasma marker, was injected 10 mg/200 μl intravenously to visualize the pial microvessels. To examine the effects of NO donor on pial microcirculation of control and PCPA-treated group, various doses of NTG were infused intravenously in each experimented group for the period of five minutes. The minimal doses that cause cerebral microvessels dilatation were 8 mg/kg BW and 10 mg/kg BW and were chosen for this experiment. The images of pial microvessels at 0 (control), 5, 15, 30 and 60 minutes post-infusion were recorded by the video system for further playback analysis. All instruments used for quantitative studies of hemodynamic parameters in the microcirculation were shown in Figure 4.2. The videotapes from each experiment were played back frame by frame and pial vessels of different diameter were randomly selected. Vessels were divided into 2 groups, 10-30 μm and 30-60 μm, based on their baseline diameter. Fifteen vessels were randomly selected from each group of five rats. Images of selected vessels were digitized and the diameters of those vessels were determined by using the computer program "Global Lab Image". The diameter of each selected arteriole was assessed by the software indicated by number of pixels (n). Then the software could convert number of pixels to micrometer. Figure 4.3 shows measurement of distance between two points (B to C) by using the position of the reference point in order to define points A to B.

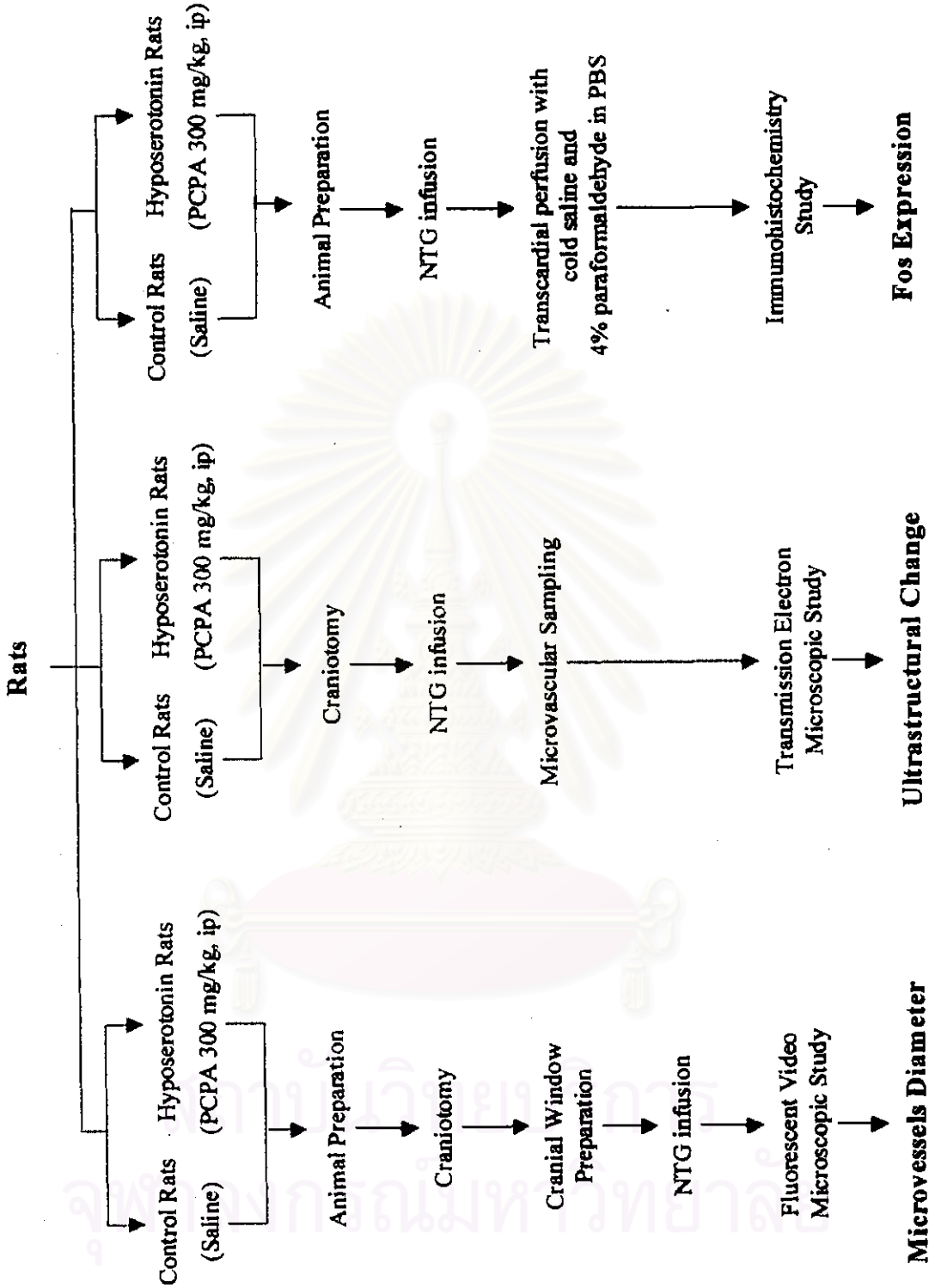

Figure 4.1. Diagram of experimental animal groups.

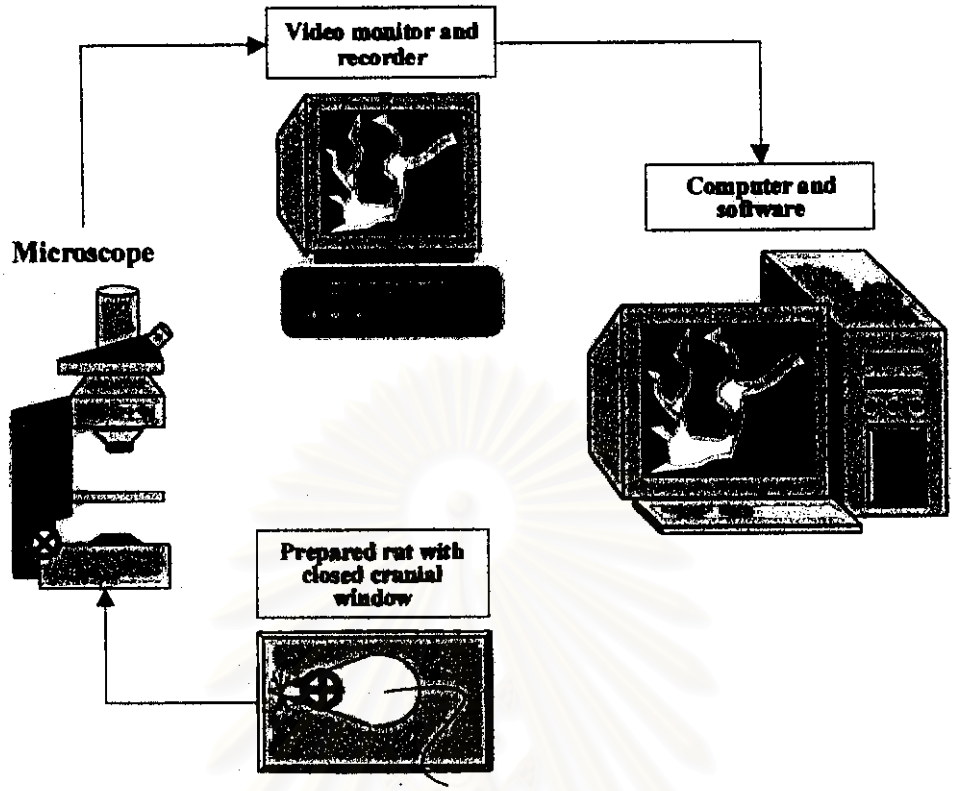

Figure 4.2. Intravital fluorescent microscopy and instruments used for quantitative studies of hemodynamic and morphologic microvasculature.

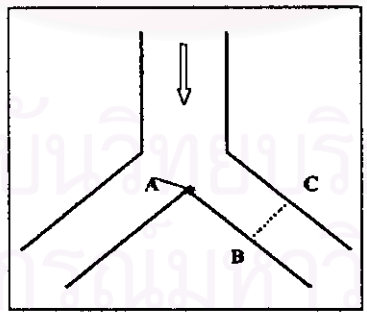

Figure 4.3. Schematic of arteriole showed the reference point A and the defined point B and C. The diameter of arteriole was measured as the length of B-C.

ULTRASTRUCTURAL STUDY

Fixation for Electron Microscopy

2 hours after NTG administration, the control rats and experimental rats were deeply anesthetized with sodium pentobarbital. Laparotomy and thoracotomy were done. 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 solution which consisted of 250 ml of phosphate buffer saline (PBS) pH 7.4 and 2 µl heparin. The effluent should run clear and then followed by 250 ml of 4% paraformaldehyde in 0.1 M. PBS pH 7.4. The right atrium was cut to permit drainage of blood and perfusates. After perfusion, the cranial window was removed and a small portion of the anterior parietal cortex containing the vessels which diameter previously measured was taken out and cut into multiple 1x1 mm cubes. The brain specimens were immediately immersed in 2.5% glutaradehyde in 0.1 M PBS pH 7.4 for at least 4 hours for further process.

Preparation for Transmission Electron Microscopy (TEM)

The tissue samples were postfix in 1 % Osmium tetroxide (OsO_4) for at least 1 hour. Tissues were dehydrated through graded series of ethanol, passed through two changes of propylene oxide, and finally embedded in plastic block (Epon 812). Semithin and ultrathin section were cut with ultramicrotome using diamond knives at 1 µm. and 500-600 A° of thickness, respectively. The semithin plastic sections were stained with toluidine blue in order to randomly select the suitable tissues area for electron microscopy. The ultrathin sections were stained with uranyl

acetate and lead citrate and were examined under a transmission electron microscope (JEOL, JEM 1210).

IMMUNOHISTOCHEMICAL STUDY

The immunostaining of nucleus-localized Fos proteins in the rat brain was performed on the free-floating section by using ABC-complex method.

Perfusion and Tissue Preparation

The rats from the control and experimental groups were perfused as described in ultrastructural study. After perfusion, the brain and cervical spinal cord were removed and postfixed in the 4% paraformaldehyde in 0.1 M PBS overnight at 4 °C. Then, the tissues were changed to 30% sucrose in 0.1 M phosphate buffer (PBS) at 4 °C for cryoprotection and were allowed to sink before sectioning. The cervical spinal cord level 1, brainstem and thalamus were cut in coronal plane by a cryostat microtome (Microm HM 50 N) at 30 μm of thickness and kept into 0.1 M PBS pH 7.4 and 4 °C until immunohistochemical staining.

Immunohistochemical Method

Free-floating sections were rinsed in PBS 2x5 min, and then placed into 1% hydrogen peroxide in PBS for 10 minutes to reduce the endogenous peroxidase at room temperature. The non-specific binding of the antibody was blocked by incubating tissue sections with 5% normal rabbit serum in PBS-A (PBS + 1% BSA + 0.3% Triton X-100) for 30 min at room temperature. Then, the section was incubated in the specific sheep anti c-*fos* antiserum diluted 1: 500 in PBS-A at 4 °C for 48 hours. After 2 days (48 hours), the sections were rinsed 3x10 min with PBS-B

(PBS + 0.25% BSA + 0.1% Triton X-100) and were then incubated with biotinylated rabbit anti-sheep IgG diluted 1:400 in PBS-B for 60 min at room temperature. After incubation, tissue sections were rinsed 2x10 min with PBS-B and then 1x10 min in PBS. The tissue sections were reacted with ABC-streptavidin horseradish peroxidase complex diluted 1: 250 in PBS for 60 min at room temperature. After 1 hour, the sections were then again rinsed 2x10 min in PBS and 10 min in 0.05 M tris-HCl buffer (pH=7.6). Finally they were reacted for peroxidatic activity in a solution containing 0.025% 3,3'-diaminobenzidine (DAB) and 0.01% H_2O_2 in 0.05 M tris-HCl buffer (pH 7.6) for 30 min. Then, tissue sections were washed 2x5 min with distilled water, mounted onto gelatinized glass slides, and coverslipped the slides with permount.

STATISTICAL ANALYSIS

The effects on pial vessel diameter produced by NTG were calculated as the maximum percent change from pre-dose baseline. All values were presented as mean±standard error of mean (SEM) and 95% confidence interval (95%CI). To compare serial changes, analysis of variance (ANOVA) for repeated measurement with posthoc Bonferroni test was employed (SPSS version 7.5 for windows). Statistical differences among various studied groups were compared using ANOVA or chi square tests for interval and categorical data, respectively. Probability values of less than 0.05 were considered to be statistically significant.