CHAPTER III

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Male Wistar rats were supplied by the National Laboratory Animal Center of Mahidol University Salaya Campus. Adult male Wistar rats weigthing 350-450 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°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.

CHEMICALS

Chemical agents

Pento barbiturate sodium (Nembutal®) was purchased from Sanofi (Thailand) Ltd. Normal saline was purchased from Hospital Products Public Co, Ltd. Fluorescein isothiocyanate-dextran (MW 150 D) (FITC), Rhodamine 6G (R6G) and Nω-Nitro-L-Arginine Methyl Ester (L-NAME) were purchased from Sigma, USA. Naratriptan hydrochloride was supported from Glaxo Wellcome Research and Development, UK. 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.

METHODS

EXPERIMENTAL DESIGN

This study was designed to investigate the effect of NO and 5-HT on CSD-evoked neurogenic vascular inflammation. The experimental rats were divided into 2 groups (Figure 3.1).

- CSD group: CSD was induced in rat brain by topical application of 3 mg of solid KCl on the surface of parietal cortex. This technique was chosen to induce CSD because of its simplicity and reducibility of results (Smith et al., 2000; Read et al., 1977; Read and Parsons, 2000; Read et al., 2000).

- Control group: 3 mg solid of NaCl was placed on the surface of rat parietal cortex instead of KCl.

To accommodate the above objectives, the study was divided into 2 parts comprising:

Study 1: To investigate the effect of NO in CSD-evoked cerebral hyperemia. The rats in KCl and NaCl groups were further divided into 2 sub-groups each.

1.1 NOS inhibitor-treated group: The rats were injected intravenously with L-NAME at the dose of 10 mg/kg BW after significant changes induced by KCl application or 10 minutes after NaCl application.

1.2 Control group: The rats were injected with physiological normal saline (NSS), intravenously after significant changes induced by KCl application or 10 minutes after NaCl application.

To investigate the dose-effect relationship of NOS inhibitor on CSD-evoked cerebral hyperemia, the rats in L-NAME-treated group were further divided into 3 sub-groups, receiving different dosage of L-NAME including: (Figure 3.2)

1.2.1 L-NAME at the dose of 1 mg/kg BW.

1.2.2 L-NAME at the dose of 10 mg/kg BW.

1.2.3 L-NAME at the dose of 100 mg/kg BW.

Study 2: To investigate the effect of 5-HT in CSD-evoked cerebral hyperemia. The rats in each group were further divided into 2 sub-groups.

2.1 5-HT_{1B} receptor agonist-treated group: The rats were injected intravenously with naratriptan at the dose of 0.1 mg/kg BW after significant changes induced by KCl application or 10 minutes after NaCl application.

2.2 Control group: The rats were injected with NSS, intravenously after significant changes induced by KCl application or 10 minutes after NaCl application.

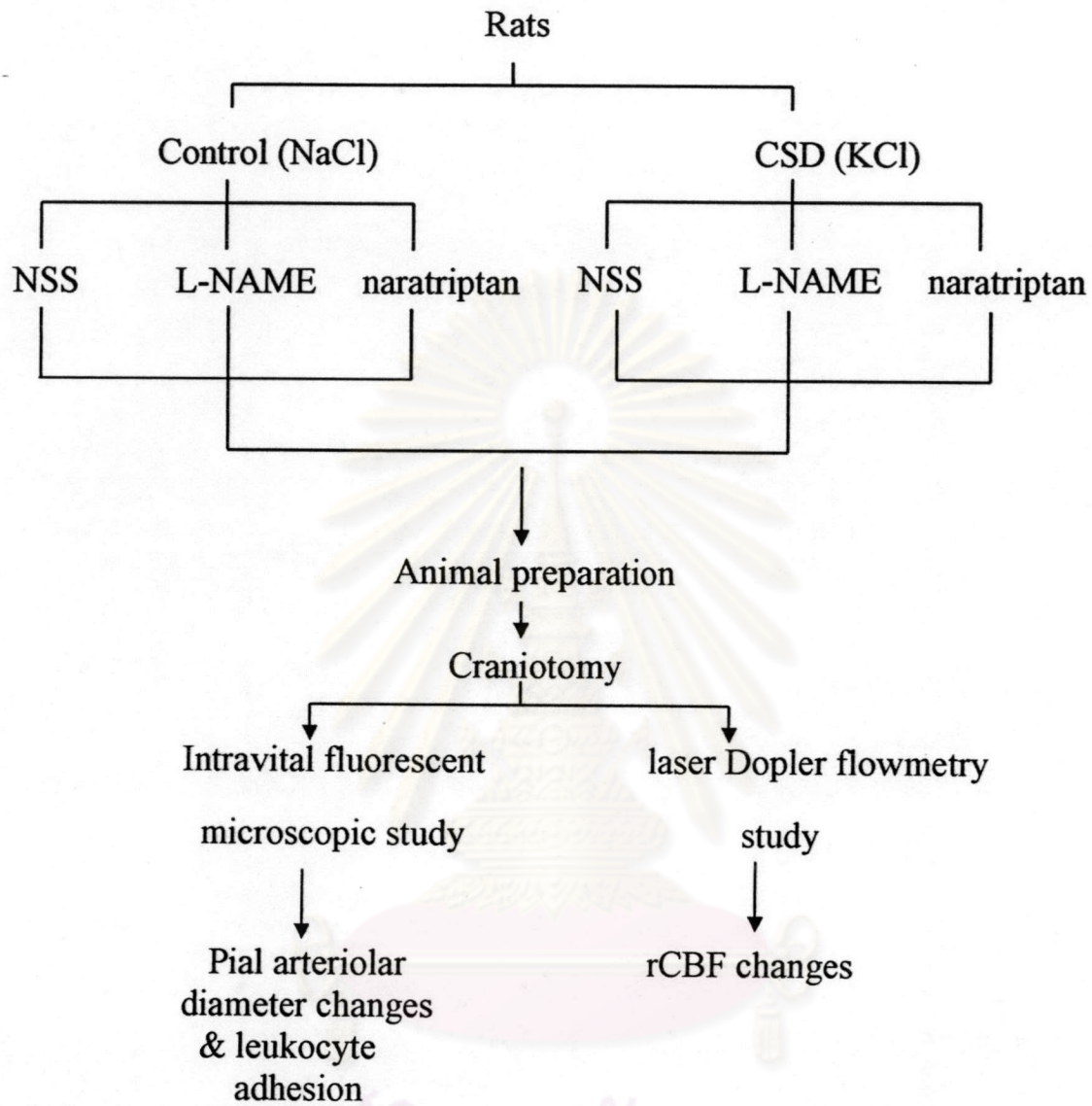

Figure 3.1 *Diagram of experimental animal groups.*

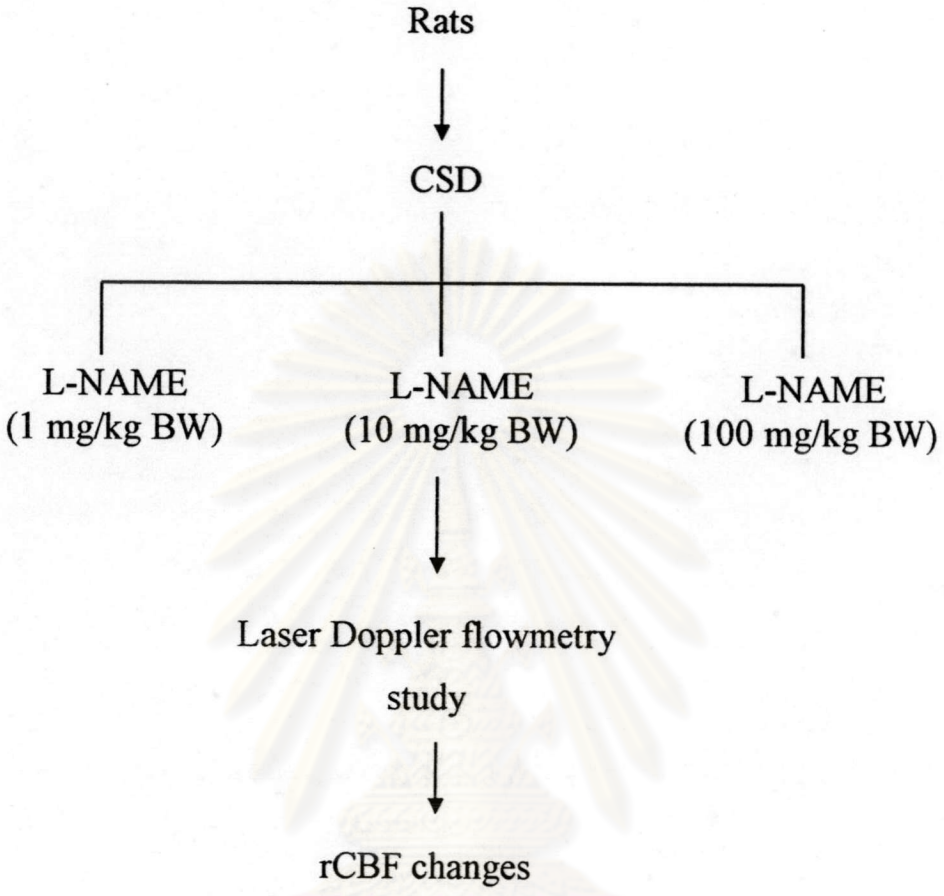

Figure 3.2 *Diagram of experimental animal groups: Dose-effect relationship of L-NAME.*

ANIMAL PREPARATION

Experimental rats were anesthetized for the duration of experiments by intraperitoneal administration of 60 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 (Nihon model TP-300T) which recorded on polygraph (Nihon RM 600, Nihon Khoden, Japan). Arterial blood was collected periodically for determination of pH, PaO_2 and $PaCO_2$ by the pH/blood gas analyzer (278 pH/blood gas analyzer, Ciba Corning Diagnostics, UK).

REGIONAL CEREBRAL BLOOD FLOW MEASUREMENT

After tracheostomy and cannulation had been performed, the rat was placed on surgical frame and the head was fixed on a head holder. A midline incision made. The right parietal bone was then exposed by mobilization of the skin either side of the incision. Two craniotomy were performed and locations were shown in the figure 4.1. The anterior craniotomy was performed in the frontal bone at 1 mm anteriorly and laterally from bregma and its diameter was about 7 mm. The posterior craniotomy was performed in the parietal bone at 7 mm posteriorly and 1 mm laterally from bregma and its diameter was about 2 mm. The anterior and posterior craniotomy opening was performed to measured rCBF changes and triggered CSD, respectively. The skull was exposed and the frontal and parietal bone were thinned by drilling with saline-cooled drill,

until the blood vessels of dura became clearly visible. The dura was opened by using microneedle. A cranial window was placed over the anterior craniotomy opening. An artificial cerebrospinal fluid was infused into the intracranial space. The fiber optic needle probe of laser Doppler flowmeter (wavelength 780 nm) (Modf ALF 21, Advance Co. Ltd., Japan) was fixed 1-2 mm above the cortical surface of the brain. The results of blood flow were recorded on polygraph (Nihon RM 6000 Nihon Khoden, Japan). CSD was induced by topical application of 3 mg solid KCl on the parietal surface at the posterior craniotomy opening (Figure 3.3).

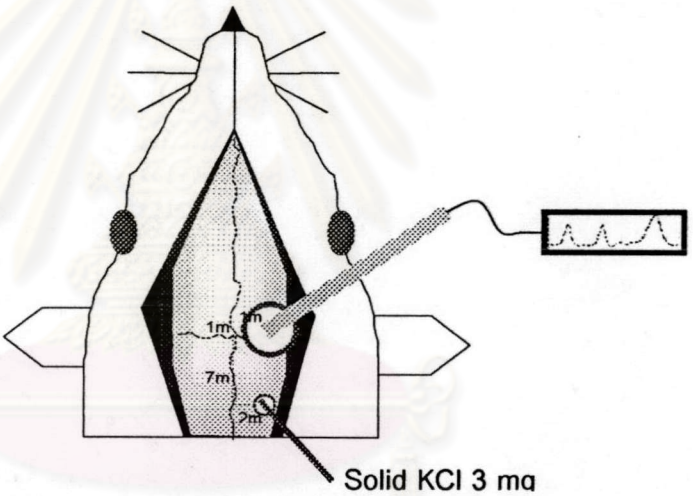

Figure 3.3 *The laser Doppler flowmetry study*

Data collection of rCBF

The rCBF was measured by using laser Doppler flowmetry and was expressed as percent change from baseline. Before induction of CSD, control observation of blood pressure, blood gas and laser Doppler flowmetry were made until a steady state was reached. After that, rCBF and MABP had been continuously recorded for 60 minutes after CSD induction and drug administration. Later, the result of rCBF had been

read at the peak of hyperemia. MABP has been read at every 5 min for 60 min.

INTRAVITAL FLUORESCEIN VIDEOMONITORING TECHNIQUE

After two craniotomy openings were performed in the same manner of the previous study using laser Doppler flowmetry. A glass cranial window was placed over anterior craniotomy opening. An artificial cerebrospinal fluid was infused into the intracranial space, continuously at rate 1 ml per hour. After placing cranial window, the animal was placed under the fluorescent microscope (Optiphot 2, Nihon, Japan) fitted with the videomonitoring system (SIT Camera, DAGE).

Measurement of pial arteriolar diameter

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. The images of pial microvessels were recorded with the video system for 60 minute after CSD induction. All instruments used for quantitative studies of hemodynamic parameters in the microcirculation were shown in figure 3.4. The videotapes from each experiment were played back frame by frame and the 2^{nd}-3^{rd} arterioles at the peak of hyperemia were selected. 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 3.5 shows measurement of distance between two points (B to C) by using point A as a reference point.

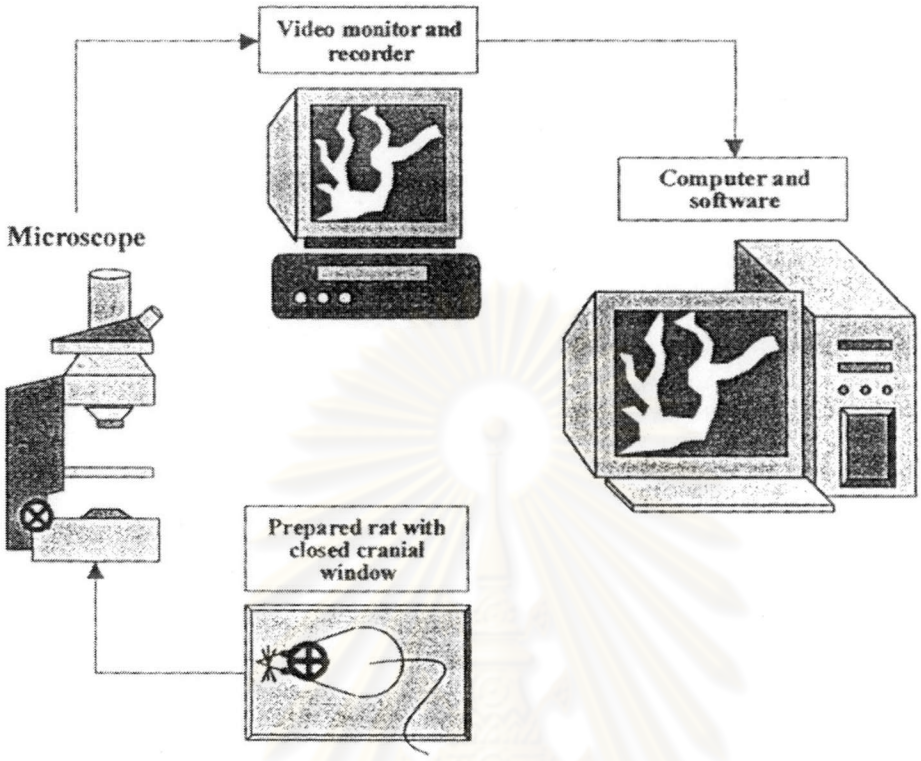

Figure 3.4 *Intravital fluorescent microscopy and instruments used for quantitative studies of hemodynamic and morphologic microvasculature.*

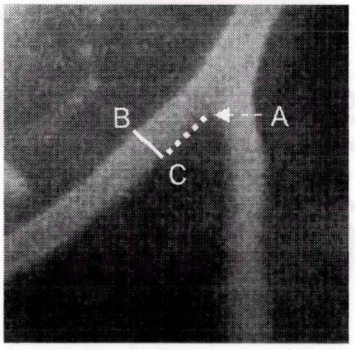

Figure 3.5 *Schematic of arteriole showed the reference point A and the defined point B and C. The diameter of arteriole was measured as the length of B-C.*

Measurement of leukocyte adhesion

To visualize the leukocyte adhesion to vascular endothelium, fluorescent marker rhodamine 6G (R6G) was administered intravenously. Adherent leukocytes in pial postcapillary venule were recorded. The emission wavelength of R6G lies between 530 and 540 nm.

During playback of the video recording, the number leukocyte adherence to endothelium of the postcapillary venule (10 to 50 μm diameter) was manually counted. The leukocyte that was counted as adherent one has to remain stationary for equal or longer than 30 seconds, the number of adherent cells were totally expressed as the number per 100 μm length the postcapillary venule.

DATA ANALYSIS

All data were expressed as mean ± standard derivation (SD). The results of rCBF and pial arteriolar diameter were presented in percent change by using ANOVA for repeated measurement with post hoc Dunnett's t-test. Probability values of less than 0.05 were considered to be statistically significant.