CHAPTER II

MATERIALS AND METHOD

Experiments performed in this study were carried on in a step-wise manners as follows:

Experimental Animals

Fifty adult male and female common tree shrews weighing between 100-150 g. were utilized in this study. All of them were taken from their natural habital in Nontaburi Province and kept in individual cage in the Faculty of Pharmaceutical Science animal house under controlled environmental conditions (12:12 light-dark cycle and room temperature). Fruits and water were supplied unlimited at least two weeks prior to experiments. Only healthy animals were chosen for experiments.

Animal Preparations

Animals were anaesthetized by intraperitoneal injection of sodium pentobarbital (Sagatal) at the initial dose of 25 mg/kg, then the anaesthetic conditions by interval small dose injections of anaesthetics. Corneal reflex, swallowing movement, rapid and forceful breathing and rigid abdomen were observed for the assesment of the level of the anaesthesia.

1. Canulation of the Femoral Artery

Skin overlying the right femoral artery was incised and the artery was exposed. Polyethylene tube (PE-50), outer diameter = 0.945 mm filled with heparinized saline (20 unit/ml) was then canulated through a small incision until its tip reach abdominal aorta. The animal was then placed in a stereotaxic apparatus for rat (Narishige SR-6) with the bite bar was set 5 mm below the ear bars.

2. Arterial Blood Pressure (ABP) and Heart Rate (HR) Measurement.

Arterial blood pressure (ABP) and heart rate (HR) were measured through pressure transducer (Elcomatic EM 751 A) and cardiotachometer (Harvard). Body temperature was maintained at 37 °C by temperature controller pad, ABP and HR were measured at normal condition and after stimulation through glass pipettes filled with KCl (3M) and biocytin (4-5%) at various positions of fastigial nucleus. Data was expressed in percentage change of ABP and HR. A positive percentage change indicates the increase, while the negative is decrease.

Experimental Procedures

Experiment performed in this study involved glass pipette preparation stimulation at various specific areas of the fastigial nucleus followed by injection of biocytin, preparation brain tissues for vibratome sectionning and reaction processes for biocytin localization.

1. Glass Pipette Preparation

Stimulation of fastigial nucleus in this study was achieved through glass capillary. The glass pipettes were prepared by pipette puller with current 19 A tips of the pipettes were observed and measured through microscope. Only long and straight ones with inner diameter = 5 μm were selected for the work. The tips were filled with biocytin 0.5 M tris-KCl. The rest of the pipetted volume were filled with 3 M KCl. The electrical impedance of the pipette was elucidate between 3-5 MΩ.

2. Stimulation of the Fastigial Nucleus

Animal was fixed in the stereotaxic apparatus with the rat ear bars,skin and connective tissues and muscles overlying the posterior occipital bone were incised and retracted laterally to expose the skull. The position of the fastigial nucleus (FN) in the cerebellum was assessed from the landmarks on the skull and dura surface following the previous work of Ware (1979) and from serial cresyl-violet-stained sections the adult common tree shrew brains area. Occipital bone was remove out by a drill and bone forcep to expose the cerebellum. The glass micropipette was mounted in a stereotaxic micromanipulator and lowered to cerebellar surface at the coordinate posterior = 2.65-3.52 mm; lateral to the midline = 0.5-1.5 mm in reference to the stereotaxic zero and penetrate bone tissues to the area of white matter anterior to rostral pole (W.ant.rFN), the rostral part (rFN), the middle part (mFN) and the caudal part (cFN) of the nucleus (Table 2).

Electrical stimulation was performed through monopolar glass micropipette as cathode, the anode was a clip attached to the scalp muscle. Stimulating pulse were generated by a square-wave stimulator (Nihon Kohden SEN-3201) and passed through a photoelectric stimulus isolation unit (Nihon Kohden SS-201 J). Stimulus current intensity was calculated from the voltage drop across a

10 KΩ resister connected in serries with the active pole of the stimulating micropipette. The voltage drop was displayed on an oscilloscope (Leader LBO-522). To localized a fastigial pressor active site, the micropipette was move downward at 0. 5 mm interval with stimulation (40-Strain of negative rectangular 0.1 ms pulse, 0.3 mA strengh). When a FPR was elicited, the micropipette was moved up and in a step of 0.1 mm interval to locate the site with the highest responses at stimulation current 0.3 mA and recorded on the oscillograph (Harvard). Photograph and diagram for the experimental set up was shown in Fig 2 and Fig 3.

3. Injection of Biocytin

After locating the FPR area; the biocytin was injected simultaneously into the area by Picospritzer (General valve corporation) (Fig 4) which connected to nitrogen gas. Pressure was set at 50 psi and duration at 25 s 4.2 pl of biocytin was set to release from the tip of micropipette at one injection. Five injections with 5 minutes time interval were performed in an experiment to confirm the release of substaintial amount of biocytin into brain tissue. Stimulations and injection of biocytin were made in the W.ant.rFN in the rostral area rFN, mFN and cFN (Table 2).

Area of FN Stimulation and injection of biocytin	Number of animals
White matter area anterior to rostral pole of FN	3
Rostral part of FN	3
Medial part of FN	3
Caudal part of FN	3

Table 2 Number of animals for experimental of FN stimulation and biocytin injection

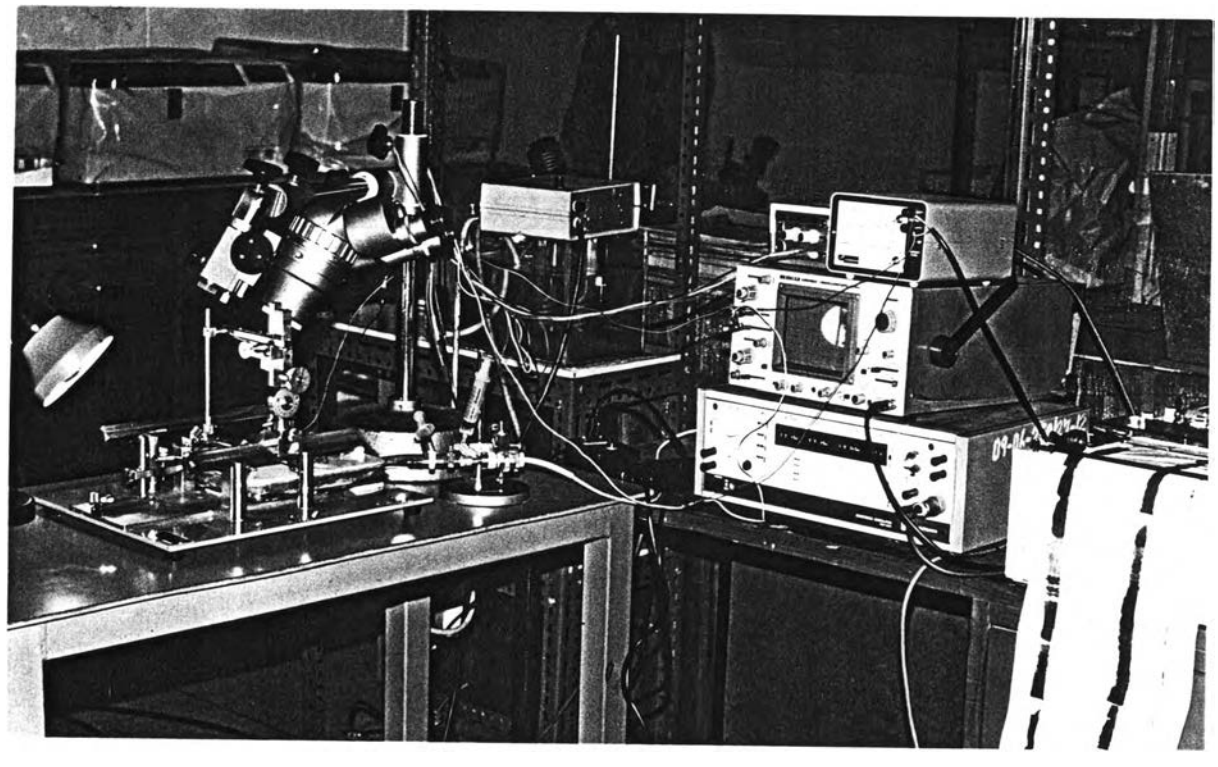

Fig 2 Photograph showing the equipment of experimental set up.

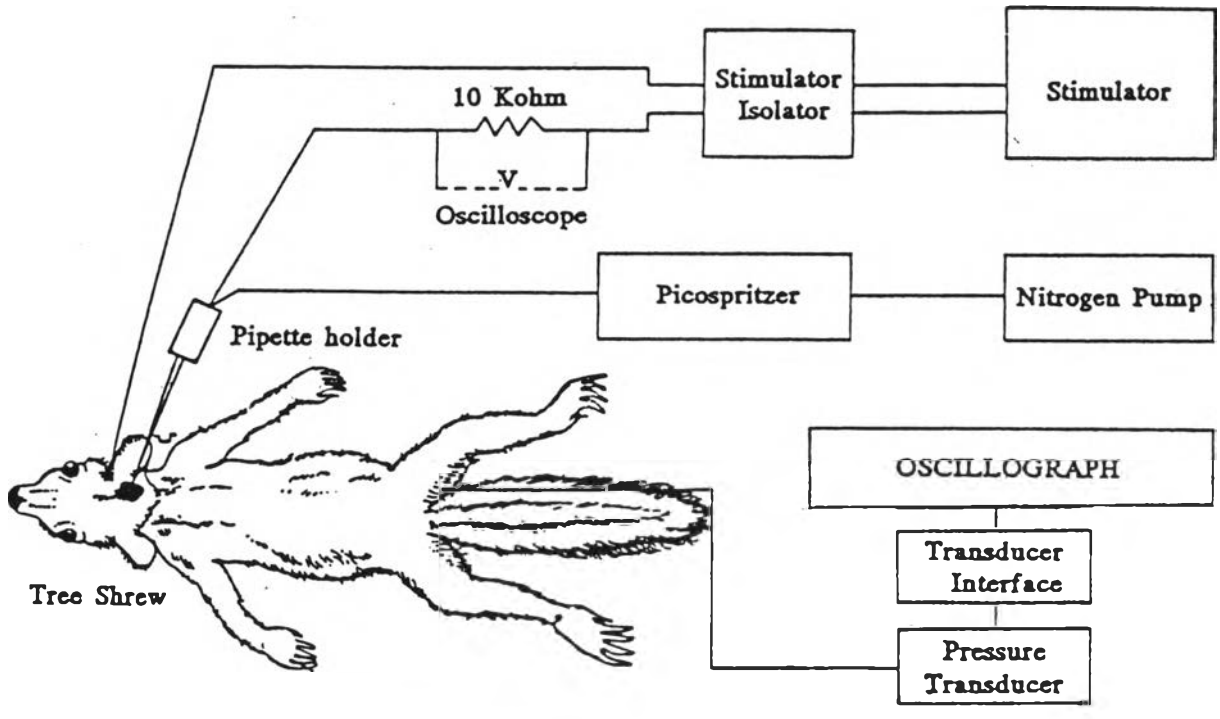

Fig 3 A diagram for the experimental set up.

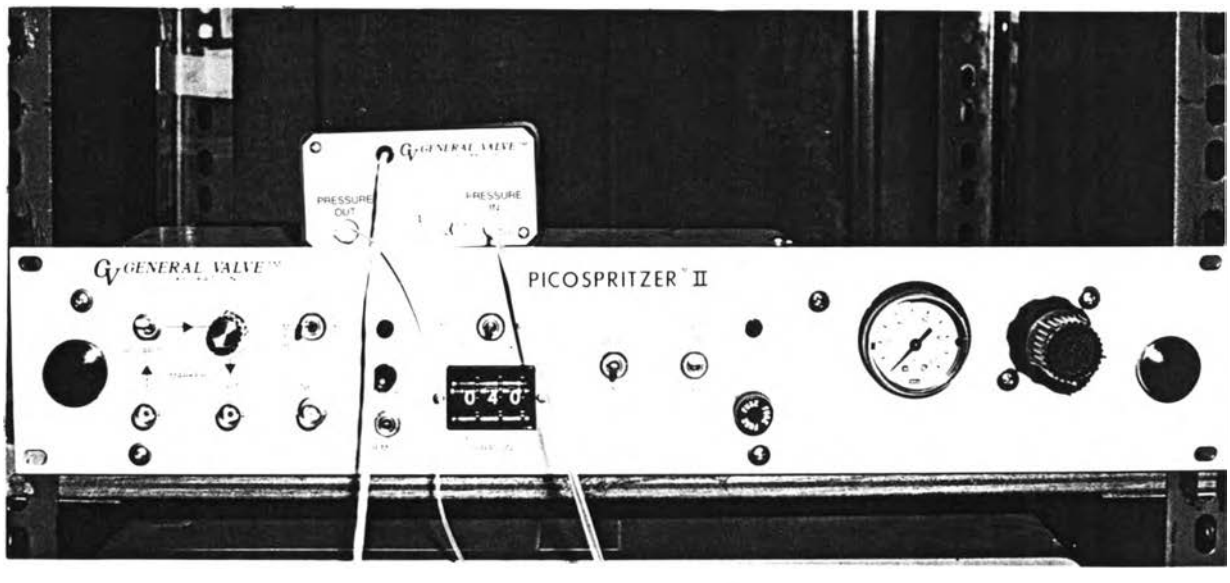

Fig 4 Photograph showing pressure injector Picospritzer (General Valve Corporation)

4. Preparation of Brain Tissue for Vibratome Sectioning

After biocytin injection, animals were kept alive for 4-16 hours. Then they were deeply an anesthetic by sodium pentobarbital (25 mg/kg) prior to transcardially perfusion with isotonic saline (pH 7.0) followed by the fixative (1-2% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). The brains were dissected quickly out of the skull and left overnight in the same fixative at 4 °C, then washed in several changes of phosphate buffer (PB). Pieces of the brain was embedded in albumin-gelatin embedding medium and serial sectioned (100 μm thickness) by vibratome.

5. Localization of Biocytin

Sections were soaked in ethanol (50%), then in ethanol (70%) containing H_2O_2 (1:1,000) and ethanol (50%) for 10 minutes each. This process was to reduce non specific endogenous peroxidase. Sections were washed in several change of PB (pH 7.4) prior to incubation with biotinylated streptavidine HRP (BSA-HRP) (dilution 1:100-1:200 in immuno diluent, Amersham International). After incubation, sections were then washed in three changes of PB (pH 7.4, 10 minutes each).

The peroxidase reaction was elucidated by incubation section in 3, 3' diaminobenzidine (DAB) containing nickle (1%) and cobalt (1%). Sections were finally in serveral change of PB (pH 7.4), mounted into chromalum-gelatin coated slides, air dried, dehydrated for microscopic observation (Fig 5). In some preparations, sections were counter strained with cresyl violet before mounting.

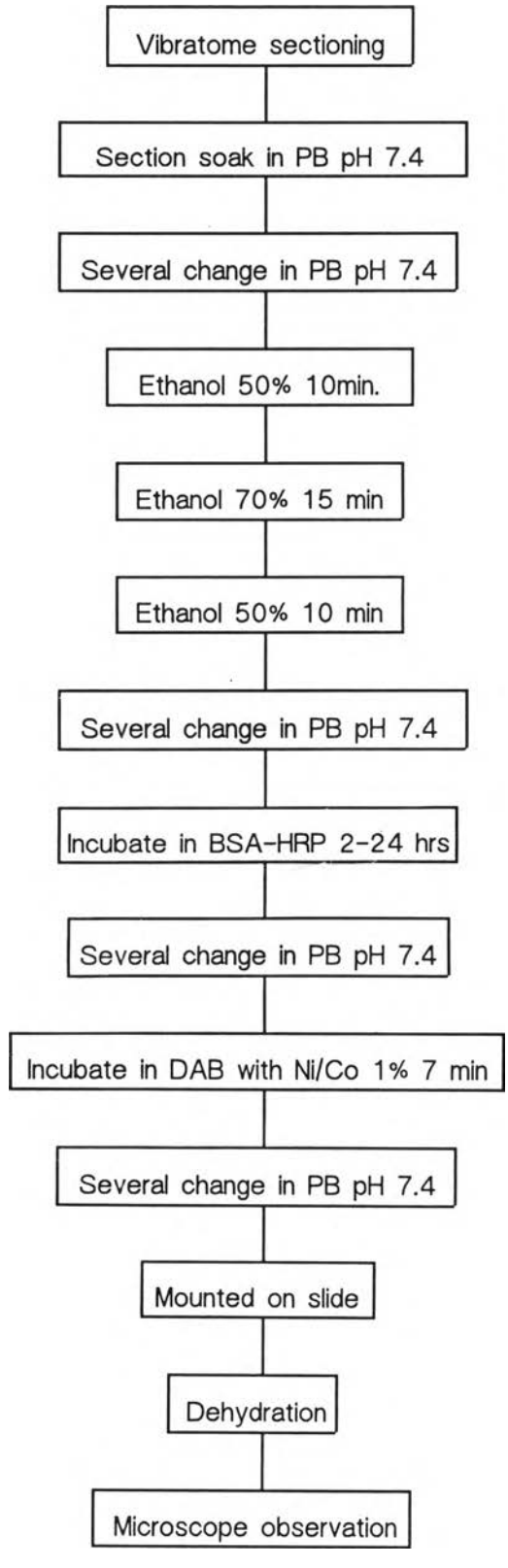

Fig 5 Diagram chart process of biocytin

Photograph of the whole sections were then taken from the processed sections. Nerve terminals and fibers were identified through microscope and photographs prior to mapping on drawings of the brain sections.

6. Statistical Analysis

Comparision of percentage change of SP, DP and MAP and HR at resting and stimulating condition in the same animal was accomplished paired t-test. P values of < 0.05 were considered to indicate statistical significance. Value are expressed as mean $\pm$ S.D.