CHAPTER III

MATERIALS AND METHOD

1. The Experimental Animal

All experiments were performed on male albino Wistar rats weighing 280-320 g, about 50 rats purchased from the National Laboratory Animal Centre, Mahidol University, Nakorn Pathom.

Animal Preparation and Surgical Procedure

The rat was anesthetized with sodium pentobarbital (40 mg/kg body weight, i.p.) with supplementary doses as required to maintain surgical anesthesia. After complete anesthesia, the rat was fixed in the stereotaxic apparatus (Narishige, Japan) then the rat's head was adjusted to align downward in 30 ° in relation to horizontal plane and rigidly secured in a stereotaxic head holder. The scalp was cut along the midline and pushed to the side to expose the skull then the neck's muscles were retracted away from occipital bone. A small hole was made through the cisterna magna to provide drainage for cerebrospinal fluid. The skull over the areas to be investigated was opened and the dura matter was removed under the dissecting microscope to expose the brain. The exposed brain surface was covered with warm normal saline to prevent drying before further electrophysiological study or microdialysis experiment. The anesthetized animal was warmed

by using a water-circulating platform with temperature maintained at 37±1° c.

2. Electrophysiological Technique

2.1 Recording Technique

For extracellular recording a single barrel micropipette was used to record bioelectric activities of vestibular nuclear neurons. The stereotaxic micromanipulator was used to advance microelectrode, filled with 4M NaCl (2-5 megaohms) into the vestibular nuclei through the opening in the skull dorsal to the cerebellum using the following coordinates : A-P = -2.0 ; L-M = 0.8 relative to the interaural line and D = 6.0 from the dura of the cortex (adapted from the stereotaxic atlas of the brain by Pellergrino, Pellergrino and Cushman, 1979). Unit activity was recorded by the microelectrode and delivered into high input impedance probe (AVZ-8, Nihon Kohden). Output from the probe was fed into a biophysical preamplifier (AVB-8, Nihon Kohden) which had filter system to minimize both low and high frequency interferences. The filtered signals were amplified and filtered with high and low cut function of a variable gain AC amplifier (AVH-10; a plug-in amplifier; Nihon Kohden). The final signals were then displayed on channel A of a dual beam memory oscilloscope (VC-10, Nihon Kohden)

2.2 Electrical Stimulation Technique

Electrical simulation was made through bipolar stimulating electrode insulated except for 0.3 mm of the tip. The

electrode was stereotaxically implanted into ipsilateral vestibular nerve using following coordinates : A-P = -1.0; L-M = 3.9 relative to the interaural line and D = 6.0 from the dura of the cortex (adapted from the stereotaxic atlas of the brain by Pellergrino, Pellergrino and Cushman,1979). Stimulating pulses were cathodic square waves which were generated by a square-wave stimulator (SEN-3201, Nihon Kohden) and delivered through one pole of electrode with the other pole connected to the reference point of the stimulator. Stimulus current was routinely monitored on an oscilloscope by measuring the voltage drop across a 10 kΩ resistor connected in series with the active pole of the stimulating electrode. Precision of stimulating electrode placement was controlled by observing typical field potentials recorded at the ipsilateral vestibular nucleus through a single barrel microelectrode. The position of stimulating electrode was adjusted until it was located at the vestibular nerve. The diagram for electrophysiological set-up was shown in Fig. 3.

2.3 Electrolytic Lesion Method

Electrolytic lesion of vestibular nerve root was produced by monopolar insulated tungsten electrode 0.2 mm in diameter with 2-3 μm sharpened tip. The electrode was stereotaxically implanted into vestibular nerve using following coordinates : A-P = -1.0; L-M = 3.9 relative to the interaural line and D = 6.0 from the dura of the cortex (adapted from the stereotaxic atlas of the brain by Pellergrino, Pellergrino and Cushman,1979). A direct current of 10 volt (1 mA) was allowed to flow for 1 min. Trial for

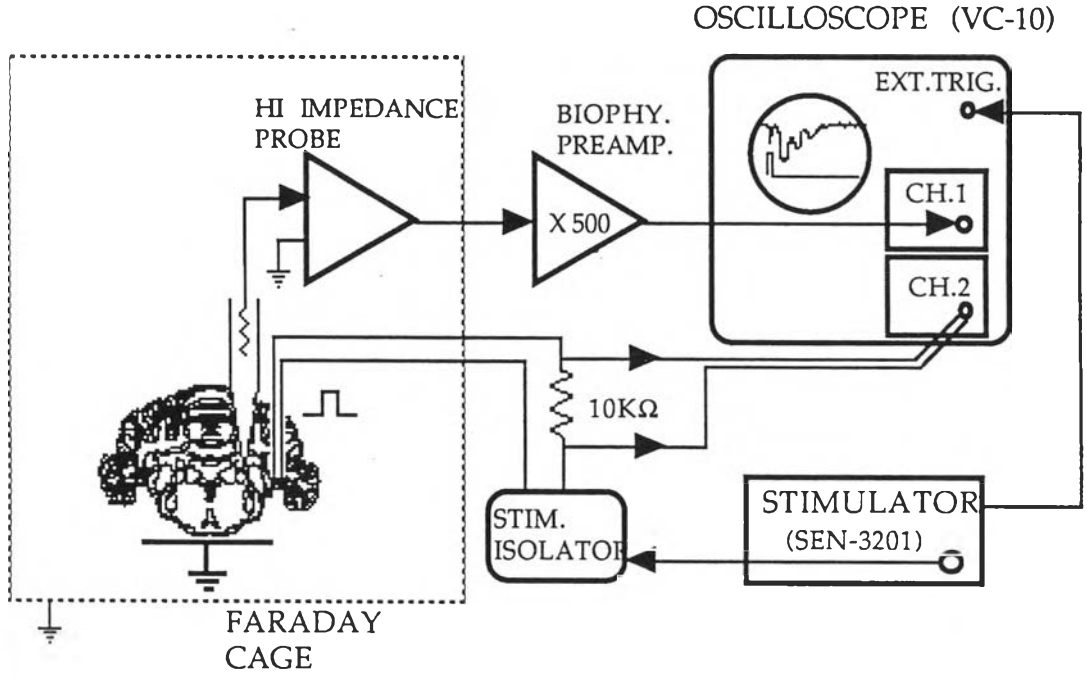

Figure 3. Diagrammatic picture of experimental set up used for electrophysiological investigation.

precision of electrode placement was carried out in a group of 4 rats whose vestibular nuclei were perfused immediately after unilateral nerve lesion. In the second and third group (5 rats each), the rat was allowed to survive 3 or 7 days prior to microdialysis experiment.

3. Microdialysis Experiment.

3.1 Preparation of Microdialysis Probe

3.1.1 The CMA / 10 Microdialysis Probe

The probe (Fig.4) was purchased from Carnigie Medicin AB (Stockholm, Sweden) which consisted of a concentric arrangement of two steel tubes. The inner tube extended beyond the distal end of the outer tube (O.D., 0.60 mm) and was covered by a tubular dialysis membrane sealed at the bottom. The fluid entered at the top through the inner tube to the bottom end from where the fluid flowed upward between the inner tube and the membrane. The dialysis between the fluid and the tissue surrounding the membrane takes placed at this point, then the fluid passed out through a side arm from which the perfusate was collected.

3.1.2 The Self-Made Dialysis Probe

In some experiments, dialysis cannula (Fig.5) was constructed from dialysis tube (Fistral 12, Hospal Industrie), 10 mm in length. The molecular weight cut off of this tube is 5000 dalton and the diameter is 200 μm. One end of the dialysis tube

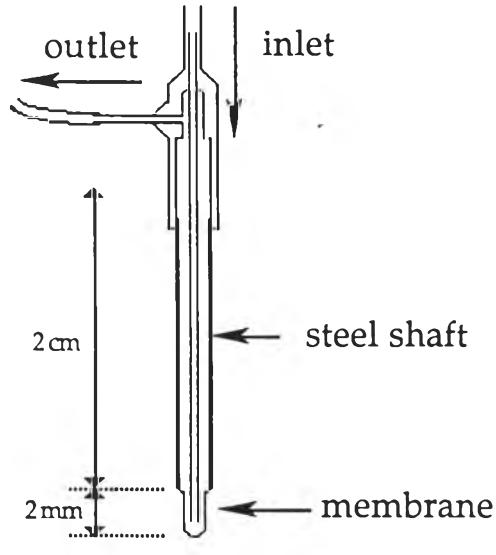

Figure 4 A schematic drawing of the microdialysis probe illustrates the concentric arrangement of two tubes. The outer tube consists of a polyether copolymeric membrane with a thickness of 60 μm and a molecular-weight cutoff of ~20,000 (Carnigie Medicin). The probe has a diameter of 520 μm and a membrane length of 2 mm. The fluid leaves the inner steel tube near the bottom of the dialysis cylender,continues up along the dialysis membrane and exits via an outer tube for collection.

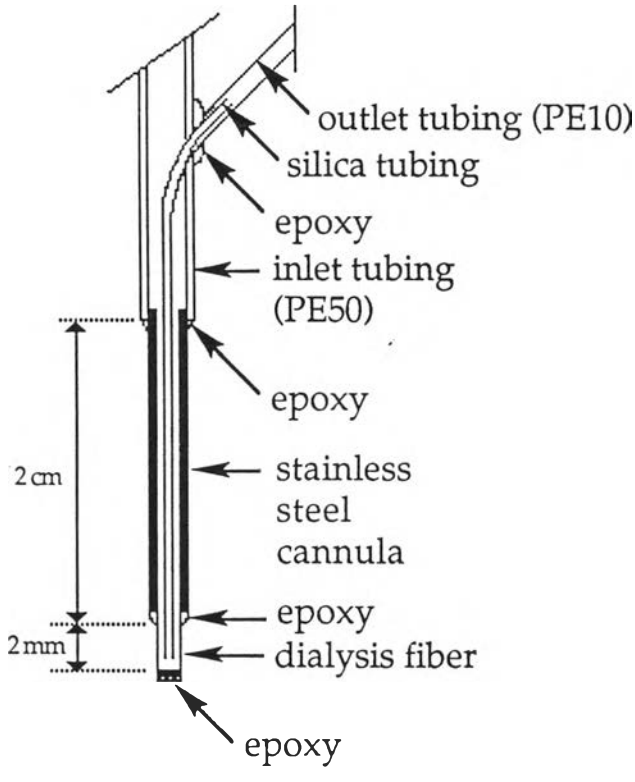

Figure5. Schematic drawing of the microdialysis probe constructed in the laboratory using a fine stainless-steel cannula to bring the fluid to the bottom of the probe covered with dialysis membrane. The fluid passes upward along the silica tube and out through the bent tube.

was sealed with epoxy (Aradrite, Ciba-Geigy). A silica tube (150 μm diameter) was inserted into the dialysis tube, then the silica tube covered with dialysis membrane was inserted into the lumen of the stainless-steel cannula (made from 25Gx1" needle). This cannula then attached with PE-50 tube and allowed a length of silica tube through the hole of PE-50 tube wall and connected with PE-10 tube. The junction of the dialysis tube, silica tube, stainless-steel cannula, PE-50 and PE-10 tube were then sealed with epoxy and allowed to dry. The total exposed length of the dialysis tube should extend approximately 0.5-0.75 mm beyond the end of the silica tube with an aid of microscopic control. For vestibular nuclei, the area of exposed membrane (i.e.,not covered with epoxy) should be 2 mm.

3.2 Instrument for Microdialysis

For running microdialysis experiment, the probe was mounted on to the micromanipulator of the stereotaxic instrument. The perfusion was controlled by the four channel continuous automatic infusion pump (Model 600-964, Harward Apparatus) with three different syringes which contained perfusion fluids of different compositions. The appropriate syringe was selected by the liquid switch (CMA /110, Carnigie Medicin, Sweden) for allowing fluid to enter and leave the dialysis probe respectively at constant perfusion speed (2 μl/min) and the perfusate was then collected from the outlet of the probe in a polyethylene tube.

3.3 Perfusion Method

3.3.1 The Perfusion Fluid

The perfusion fluid for this microdialysis experiment was artificial cerebrospinal fluid (aCSF) which contained an identical ionic compositions as that of the fluid in the brain interstitial space. The compositions of aCSF were NaCl 120 mM, $NaHCO_3$ 15 mM, KCl 5mM, $CaCl_2$ 15 mM, $MgSO_4$ 1.0 mM and glucose 6.0 mM. The pH was adjusted to 7.3 by aerating with O_2-CO_2 (95-5 v/v) mixture. In some experiments, potassium ions in the perfusion fluid were elevated to 100 mM (high-K^+ CSF) with Na^+ content reduced by the same molar equivalent of the increased K^+ to retain osmolality.

3.3.2 *In vitro* Testing

Before implanting the probe into the brain, each probe was tested *in vitro* in standard solution in order to test the properties of the membrane and to determine the recovery of amino acids. A probe was mounted on a holder and lowered in the small beaker filled with solution of 10 nM of each amino acid (Asp, Glu, Ser, Gln, Gly, Tau, Ala and GABA). The flow rate was adjusted at 2 µl /min and the sample collected after 30 min of perfusion. Recoveries of substances into the probe were expressed in relative recovery which is the ratio of the concentration of a substance in the perfusate to its concentration outside the membrane. These values were used to correct the *in vivo* data to produce a value which reflected the extracellular

concentration of the amino acids in the vestibular nuclei of the rat.

3.3.3 *In vivo* Experiment

The *in vivo* experiment(Fig.6) was performed on the anesthetized rat which was prepared as previously described. A microdialysis probe was stereotaxically implanted into the vestibular nuclei area using following coordinates : A-P = -2.0 ; L-M = 0.8-1.0 relative to interaural line and D = 6.0 from dura of the cortex (adapted from the stereotaxic atlas of the brain by Pellergrino, Pellergrino and Cushman, 1979) and started perfusing with aCSF at the flow rate 2 µl/min. After implantation, the first two fractions were discarded to avoid effects of the implantation so that the steady state level was reached and then the perfusate fractions were collected during eight consecutive 15-min periods.

4. Amino Acids Assay

Aliquots of perfusates (30 µl) were analyzed for eight amino acids according to the method of Lindroth & Mopper (1979) using a high performance liquid chromatography (HPLC) with fluorescence detection following pre-column ortho-phthaldialdehyde (OPA) derivatization.

4.1 Apparatus

The HPLC system consisted of seven components, i.e. pump, solvent conditioner, HPLC controller, column, column

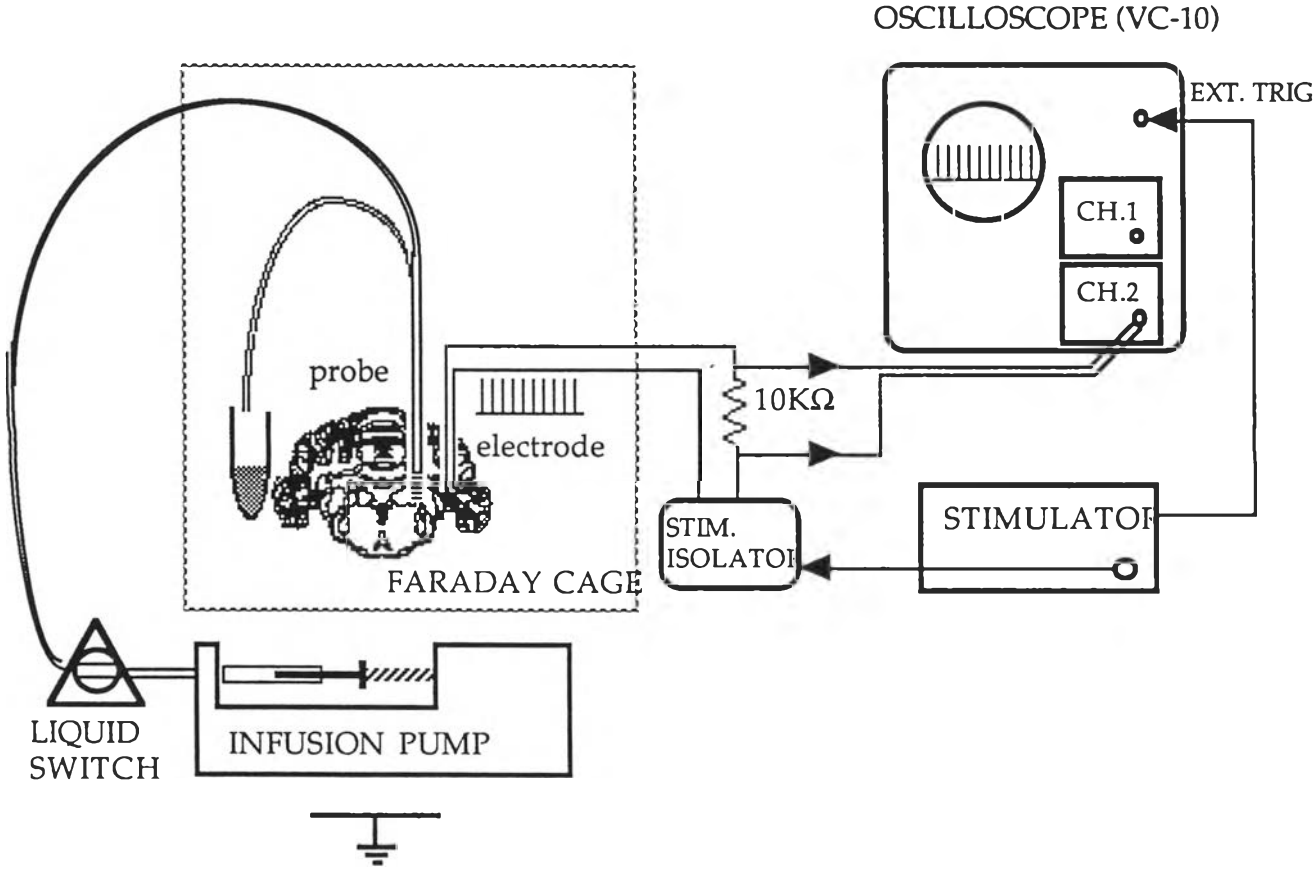

Figure 6 Diagrammatic picture of experimental arrangement employed in experiments involving nerve stimulation and microdialysis experiment.

oven, fluorescence detector and integrator. A solvent delivery pump (Model 2150, LKB, Sweden) was used for delivery of the mixture of two solvent (phosphate buffer: methanol) which was degassed continuously by aeration with helium by using solvent conditioner (Model 2156, LKB). HPLC controller (Model 2152, LKB), a central processing unit, was used for precise and accurate gradient elution and HPLC system control. An analytical column (Ultropac HPLC Column, TSK ODS-120T, 250x4.6 mm, 5 µm particle size, LKB) was protected by guard column (Upchurch Scientific Inc., 30 x 4.6 mm I.D.,5 µm particle size). The temperature of the analytical and guard column were controlled by HPLC column oven (Model 2155, LKB). The column effluent was monitored by the scanning fluorescence detector (Water470, Millipore Corporation, USA) at an excitation wavelength of 330 nm and emission wavelength of 418 nm. The area of each chromatographic peak was automatically determined by a computing integrator (Model SP4270, Spectra Physics). The diagram of the HPLC system was shown in Fig. 7.

4.2 Reagent and Chemicals

Chemicals were obtained from the following sources : absolute ethanol and 2-mercaptoethanol were obtained from Merck; disodium hydrogen phosphate, sodium dihydrogen phosphate and sodium hydroxide from Riedal-Haen; boric acid and methanol (HPLC grade) from BDH chemical; ortho-phthaldialdehyde and standard amino acids from Sigma

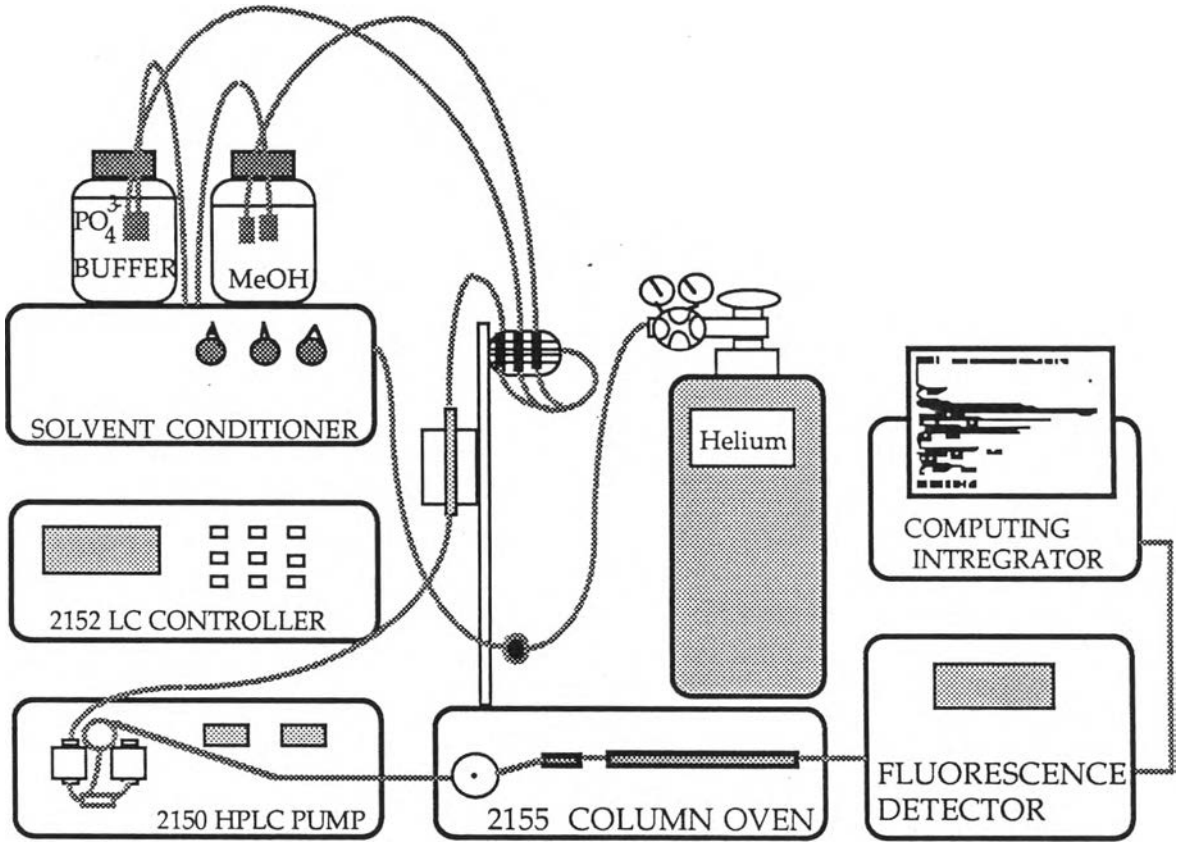

Figure 7. Diagrammatic picture of HPLC system

Chemical Co. All reagents and solvents were of analytical grade and used without further purification.

4.3 Chromatography

The mobile phase was composed of 0.01 M phosphate buffer, pH 7.3 and methanol (HPLC grade). For the phosphate buffer, the pH was set by mixing equimolar solutions of Na_2HPO_4 and NaH_2PO_4 in different proportions. The mixture was then filtered through a 0.22 μm membrane filter (Millipore, USA) under vacuum.

For gradient run, the mobile phase gradient was increased from 20% to 65% methanol in one linear step at the increment rate of 2%/min. The solvent was pump through the column at a rate 0.5 ml/min which yielded a pressure of 110-120 bar. At the end of the run, the mobile phases were changed back to 20% methanol as initial conditions. The sample was applied through a 50 μl loop to the column with an injection valve. The column temperature was maintained at 30°c.

4.3.1 Preparation of OPA-Amino Acids

Precolumn amino acid derivatization was accomplished by mixing 30 μl of a perfusate sample or standard amino acids with 60 μl of OPA-thiol reagent for exactly 2 min prior to injection into the HPLC system. The reaction of OPA-thiol with an amino acid was shown in Fig. 8.

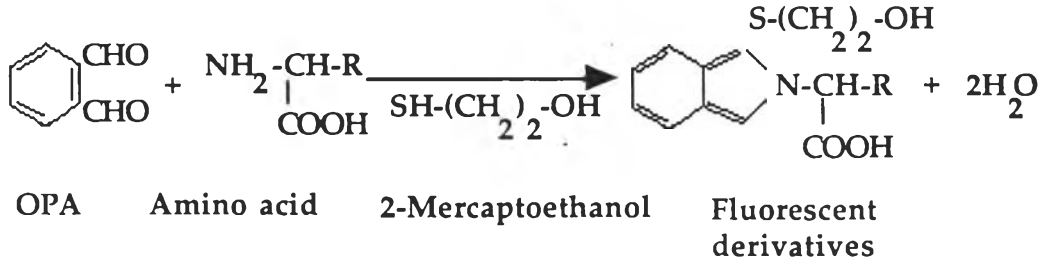

Figure 8. o-Phthaldialdehyde (OPA) forms fluorescent derivatives in aqueous solution when reacts with amino acids in the presence of the reducing agent 2-mercaptoethanol (derived from Lindroth & Mopper, 1979).

4.3.2 Preparation of OPA-Thiol Reagent

The OPA-thiol reagent was prepared by dissolving 27 mg of OPA in 5 ml of absolute ethanol. To this solution, 100 µl of 2-mercaptoethanol were added and then diluted up to 10 ml with 0.4 M boric acid adjusted to pH 10.4. The reagent mixture was allowed to age for at least 24 hrs. prior to use. The reagent strength was maintained by addition of 20 µl of 2-mercaptoethanol every 3-4 days.

4.4 Standard Solutions

Amino acids stock solution containing Asp, Glu, Ser, Gln, Tau, Ala and GABA were prepared by dissolving them in 10 ml of 50:50 mixture of triple distilled deionized water and methanol to give concentration 0.1 µmol/50µl. These solutions remained stable for several months if kept refrigerated. Working standard solution was prepared by diluting the stock solution to give concentration 10 nmol/50 µl with triple distilled deionized water. These standards were derivatized as previously described and analyzed before running microdialysis experiment to asses reagent stability and chromatographic consistency.

4.5 Peak Identification and Quantification

Data were collected on a computing integrator at chart speed of 0.25 cm/min. Peaks were identified by comparing the retention time of each peak in the sample solution to that of individual peak in standard solution. The concentration of each amino acid was then calculated using the response of fluorescence

detector, provided that standards and samples have been run under the same conditions. Amino acid contents of perfusate sample were expressed as pmol/50 µl (mean ± S.E.M.).

5. Histological Study

At the end of each experiment, both of stimulation site and the position of microdialysis probe were examined histologically. Stimulation site was marked electrolytically by passing direct current 1 mA for 1 min through the electrode. The rat was perfused intracardially with 200 ml of physiological saline followed by 100 ml of 10 % formalin-saline. The brain was removed from the skull immediately following perfusion and stored in 10% formalin for 24 hrs, then placed in a 10% formalin-20% sucrose solution for 48 hrs prior to sectioning. Frozen sections of the brain were obtained at the thickness of 40 µm by using a freezing microtome. The sections were stained with cresyl violet and studied for the tissue damage in vestibular nuclei caused by microdialysis probe and lesion track caused by DC marking current in vestibular nerve root.

6. Statistical Analysis

Experimental data were expressed as mean ± S.E.M. Statistical significance was tested according to Student's t-test for paired or unpaired varieties. The p-values less than 0.005 ($p<0.005$), 0.05 ($p<0.05$)and 0.01 ($p<0.01$) were accepted as being statistically significant.