CHAPTER III

MATERIALS AND METHODS

The Experimental Animals

60 male and female common tree shrews (*Tupaia glis*), weight of each was about 120-160 grams, were used in this study. Animals were normally housed in groups of two in rodent cages and located in a room temperature on standard light-dark cycle. Before commencing the study, common tree shrews were habituated to handling. Food and water were provided ad lib.

Animal Preparations and Measurements

1. Measurements of Electromyographic (EMG) Activity : Before SN lesion

The electromyographic (EMG) activity was recorded from the triceps surae (gastrosoleus muscle) of non-anesthetized animals, at rest. Pre-drug control group, EMG activity was investigated as reference for the determination of

changes in the resting-EMG activity after substantia nigra-lesion, and after electrical cerebellar stimulation. The EMG activity was recorded with pairs of percutaneous inserted telflon insulated stainless steel fine wire electrode (Bipolar needle electrode, needle length 20 mm, needle diameter 0.41 mm, core area 0.024 mm^2, cutting angle 15˙, NM-220T). Animals were placed individually in body-fixator and the EMG activity of their hind limbs was recorded continuously and calculated at rest. The electrical signals were amplified, band-pass filtered (5 Hz - 10 Hz). The MEM-3202 eletromyograph (Nihon-Kohden) was free from AC interference during examination by a high performance preamplifier and AC interference filter.

2. Unilateral Substantia Nigra-Lesion.

After measurements of normal EMG activity at rest, the animals were anesthetized with pentobarbital sodium. An initial doses of 25 mg/kg body weight was administrated intraperitoneally and additional doses of 2-10 mg/kg as required. After being mounted on a stereotaxic apparatus for rat (Narishige SR-6) in a prone position with the bite bar set 5 mm below the ear bars. The scalp was incised. A dental drill was used to place a single burr hole at the appropriate site on the left. Selective lesions of substantia nigra were created by injecting 6-OHDA using a Hamilton 10 µl syringe with a needle (480 mm

OD.), according to the following coordinates relative to apex : 1.0 ± 0.2 mm anteroposterior level (AP), and 1.0±0.5 mm lateral to the stereotaxic zero reference point and 10.0±0.5 mm depth from the surface of dura matter, which was assessed previously from serial cresyl violet-stained sections of the tree shrews brain. The 6-hydroxydopamine HBr (6-OHDA, sigma chemical Co.) was dissolved in 0.1 mg/ml ascorbate-saline freshly solution at a concentration of 10 μg in 2 μl (calculated as free base) added to prevent auto-oxidation. This solution was kept on ice until it was injected through a Hamilton microsyringe at a rate of 1 μl/min plus 3 min diffusing time. An antibiotic power was applied locally before suturing. Two animals received injections of 6-OHDA on the right substantia nigra for confirmation of lesioned side.

3. After Substantia Nigra - Lesion.

Twenty-four to fourty-two hours after substantia nigra lesions, animals were circling around the body axis and postural asymmetry. Abnormal muscle tone was observed and assessed by passive leg movements, and EMG finding was recorded of both side of triceps surae, like step 1.

4. After SN-lesion with Cerebellar Electrical Stimulation

4.1. Preparation of bipolar stimulating electrode

The tungsten wires electrodes, 1-2 μm tip diameter was shaped by electrochemical ething that applying 10-15 V AC voltage between wires electrode and carbon rod in 20 % sodium hydroxide solution and washing it with distilled water. The tungsten wire electrode was insulated with varnish and the insulated was removed from the tip of electrode between 10-20 μm by applying 90 V AC voltage, frequency 50 Hz to the tip of electrode that passed into 9 % NaCl solution. Two electrodes were matched with epoxy, distance of tip to tip between two electrodes about 0.2 - 0.4 mm The electrical impedence of the electrodes is between 2 - 3 megaohms.

4.2. Cerebellar Electrical Stimulation

The SN-lesioned animal was anesthetized and fixed in the stereotaxic, a suboccipital craniectomy was performed. The bipolar electrodes were placed stereotaxi- cally over the vermis zone (C1, C2, C3), left intermediate zone (L1, L2, L3) and righ intermediate zone (R1, R2, R3) of anterior cerebellar surface. The preliminary map had been drawn (Figure. 2) and recorded coordination point (Table 2). Confirmation of position was obtained by stereo-microscope. The current was applied to

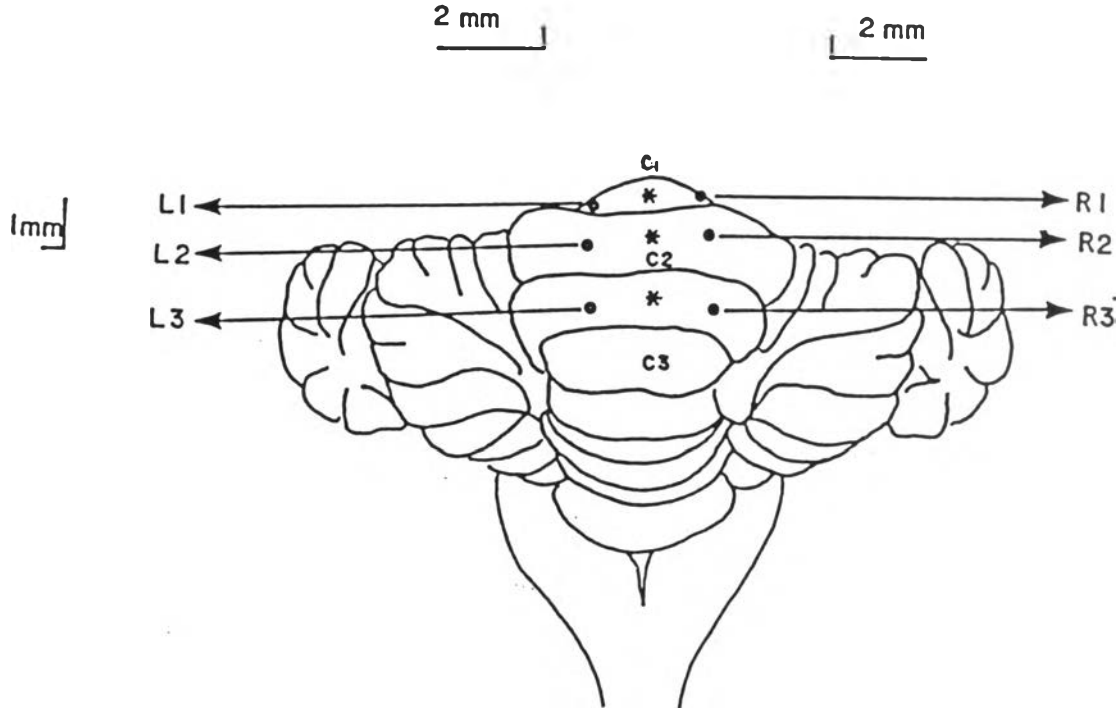


Figure 2. Superior surface of cerebellum of tree shrew. The stimulated sites lie on vermis part (C1,C2,C3), left part (L1,L2,L3) and right part (R1,R2,R3) of anterior lobe. Transverse zone shows lobule I (L1,C1,R1), lobule II (L2,C2,R2) and lobule III (L3,C3,R3)

Part of cerebelllum	Location of electrode	Coordination relative to stereotaxic zero reference		
		Posterior (mm)	Left/Right : (mm)	Vertical : (mm)
I. Vermis part	C_1	3.2-3.5	zero	0.1-0.5
	C_2	4.2-4.5	zero	0.1-0.5
	C_3	5.2-5.5	zero	0.1-0.5
II. Left intermediate part	L_1	3.2-3.5	1.9-2.0	0.1-0.5
	L_2	4.2-4.5	1.9-2.0	0.1-0.5
	L_3	5.2-5.5	1.9-2.0	0.1-0.5
III. Right intermediate part	R_1	3.2-3.5	1.9-2.0	0.1-0.5
	R_2	4.2-4.5	1.9-2.0	0.1-0.5
	R_3	5.2-5.5	1.9-2.0	0.1-0.5

Table 2. Coordination of stimulated sites relative to stereotaxic zero reference point.

the cerebellar surface, the active electrode was connected to the cathode pole of a current generator, and the inactive electrodes to the anode pole. Stimulating pulse were generated by a square-wave stimulator (Nihon-Kohden SEN-3201) and passed through an isolation unit (Nihon-Kohden SS-201J). Stimulus current intensity was calculated from the voltage drop across a 10 kilo-ohms resister connected in series with the active pole of the stimulating electrode, the voltage drop was displayed on an oscilloscope (Leader LBO-522). Current application of duration could be varied from 0.02, 0.05, 0.1, to 0.2 ms and various frequencies of 1, 10, 50, 100 and 200 Hz. The amplitude of applied current was increased at a rate of 0.05 mA per 40 ms.

The animals received stimulation for 40 seconds, which the stimulation parameters were defined in an effort to suppressing EMG response. The EMG change of both triceps surae were observed and recorded before, during, and after stimulation in non-anesthetized animals. The hindlimbs were in an intermediate position. Latency period (sec) was duration between the starting stimulation until the suppression EMG response that was recorded. And the after effect period was recorded when the stopping stimulation until the suppression EMG effect disappeared. The effort stimulus parameters (i.e. frequency, duration and current intensity) were recorded.

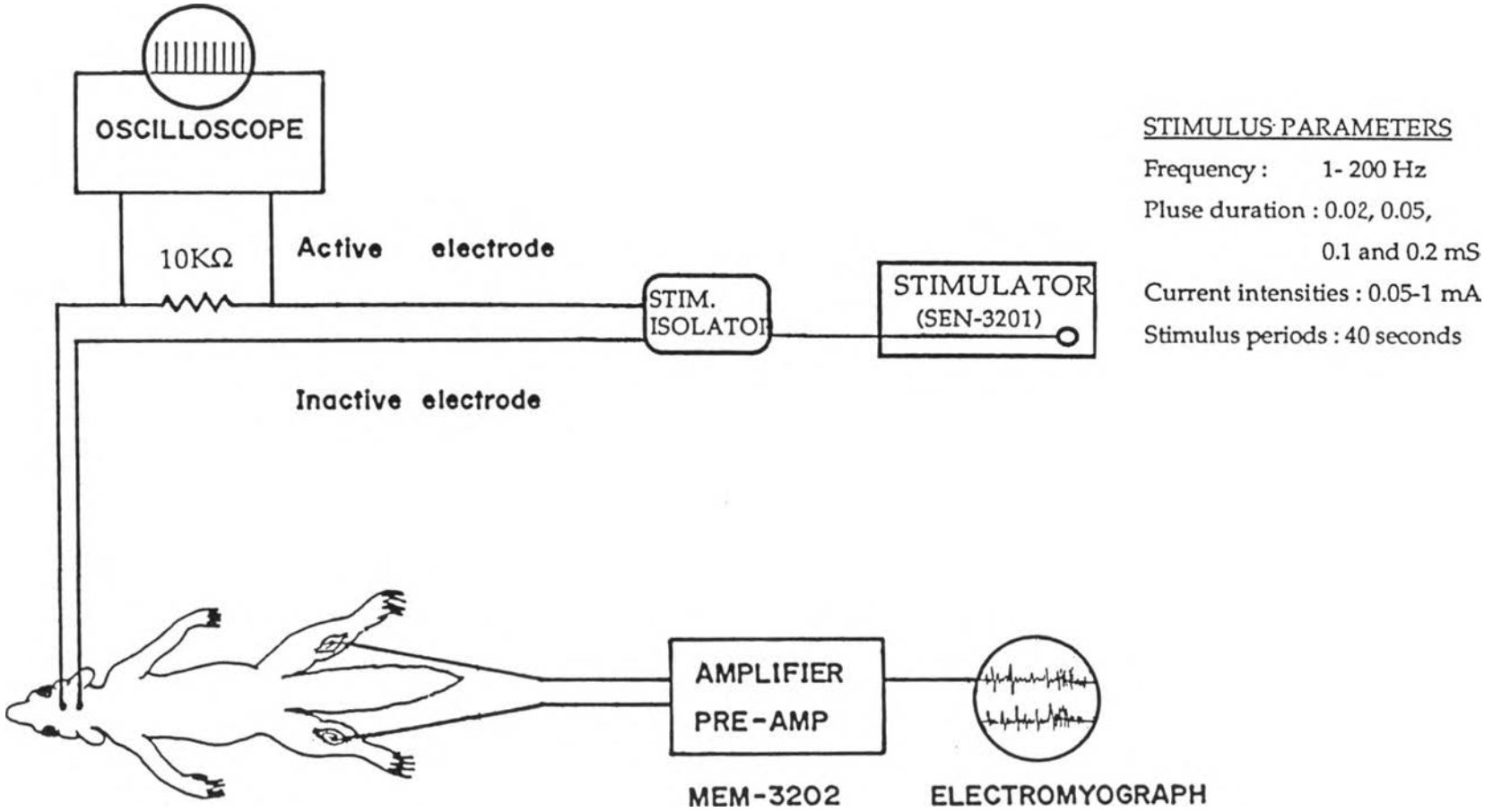


Figure 3. Diagrammatic picture of experimental set up, used for electrophysiological investigation

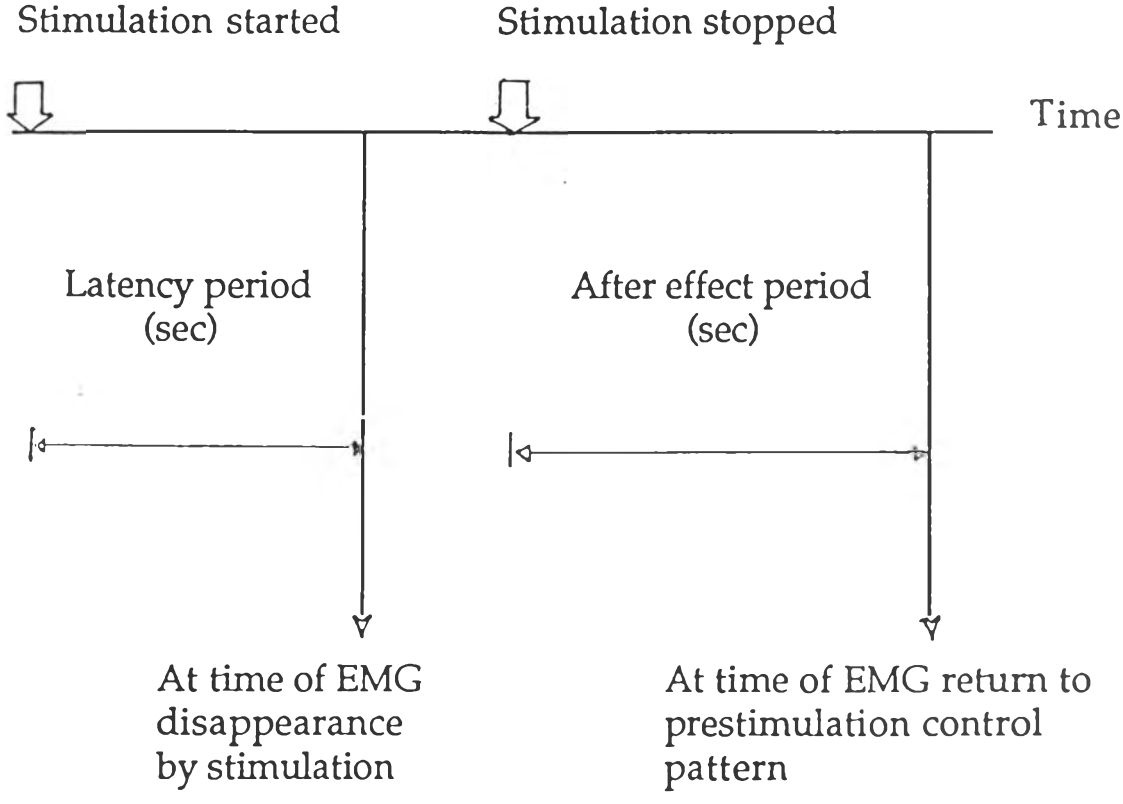


Figure 4. Diagrammatic representation of latency period (duration from starting stimulation to disapperance of EMG) and after effect period (duration from stopping stimulation to rebound of EMG

Verification of Stimulation Position and Lesion Site

1. Stimulating Electrode Marking

For histology confirmation, at the end of each experiment, the site of stimulation (C_2) was routinely marked by small electrolytic lesions through the same electrodes. The lesions were produced by passing a DC current of 1 mA, for 60 seconds

2. Fixing the Brain Tissue

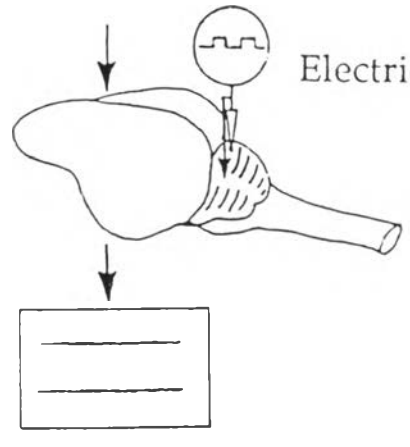
At the end of electrolytic lesion, the animals were deeply anesthetized with sodium pentobarbital. They were perfused intracardially with 150 ml of physiological saline solution until the blood was completely washed out the vascular system. Then, they were perfused with 4% formalin, in 0.1 M phosphate buffer at pH 7.4. The skull was opened, the extracted brain was placed in 4% formalin for 72 hours and transfered into a solution of 30% sucrose in the same phosphate buffer for 48 hours prior to sectioning.

3. The Neuroanatomical Studies of Lesioned and Stimulated Sites

Serial coronal frozen section of the brain were obtained at the thickness of 40 μm by using a freezing microtome. The brain sections were picked up onto microscopic glass slides and stained with cresyl violet. They were mounted under the coverglass. The lesioned site and stimulated site were investigated by microscope.

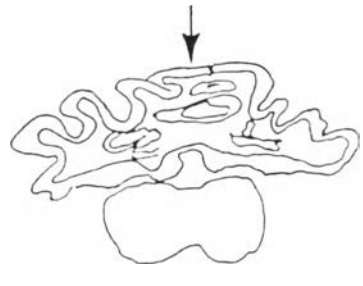
EMG studies of both triceps surae at rest

EMG studies of both triceps surae at rest before stimulation



EMG studies after stimulation

Measurement : Latency period (time of started ES to time of suppressing effect)
: After effect period (time of stopped ES to time of EMG rebound)



Stimulation site

Figure 5. Diagrammatic shows the step of the experiment