CHAPTER II

General methods

Experimental Animals and Anaesthesia

Experiments were performed on adult albino rats (Wistar strain) of both sexes weighing 190-300 g. All electrophysiological investigations were conducted on rats anaesthetised with urethrane. The animal recieved a single intraperitoneal injection of 1.5 $g.kg^{-1}$ of urethrane (ethyl carbamate)

Steriotaxic Techniques

The so-called stereotaxic technique was introduced for the first time by Clarke and Horsley in 1906, after which it has been considered essential for achieving precise placement of electrodes or cannulae in many experiment manipulation (i.e. electrophysiological recording, lesioning, chemical injection and stimulation) of the central nervous system (CNS). In principle, electrode or cannulae are introduce into specific area by reference to three dimentional system of co-ordinates determined by external land markes on the skull or by other reference points from pre-existing atlas

The animals were mounted on a Narishige's stereotaxic apparatus. The animal's head was rigidly secured in a conventional stereotaxic head holder. The upper jaw bar and two ear bars were aligned in the same horizontal plane.

Surgical Procedure

After fixing the animal in the stereotaxic apparatus a midline incision was made in the scalp from the level of fronto-nasal suture to the neck. The scalp was reflected to expose the skull, and attached muscle scraped away. The musculature covering the nape was clear to expose the cisterna magna. To mimimize cerebral edema a small incision was made through the cistern to provide a drainage for cerebral fluid. The dorso-caudal surface of the cerebellum were exposed by craniotomy and the dura matter removed under microscopic control to expose the brain. Care was taken from this stage to prevent the exposed tissue from drying by irrigating the surface with mixture of liquid parafin and vaselin.

Before insertion of the microelectrodes, a small patch was then made in the pia matter to facilitate the insertion of glass micro-electrodes. This step was considered neccessary since advancing a microelectrodes through and intact pia matter tended to cause blockage. Moreover, after insertion of the electrodes, the exposed brain surface was covered with layer of 4% agar in saline to minimize pulsation which might interfere with electrophysiological observation.

Microiontophoretic Techniques

The technique was first employed by Suh, Wang and Lim (1936) when they were successful in producing the rise in blood pressure in experimental animal by iontophoretic ejection of acetylcholine from a 100 µm. microelectrode into the floor of the fourth ventricle. However, not until more recently that other workers had turned their attention to this method (del Castillo and Katz 1955). In particular,

this technique has been developed to successful extend by the Canberra group (e.g. Curtis and Eccles 1955).

Microiontophoresis is a method of providing a controlled application of chemical substance from fine microelectrode or micropipette by passing of electric current. The term is sometime interchangable with microelectrophoresis, but in a more strict sense the microiontophoresis should be applied only when describing the movement of ions by current flow. Thus, in microelectrophoresis, microiontophoresis occurs, which usually is associated with electro-osmosis (see below).

Microelectrode

In most cases when microiontophoretic application of substances was performed in this study seven-barrel microelectrode was used. Seven-barrel microelectrode was fabricated in the laboratory from borosilicated glass tubing (external diameter 1.55 mm $\pm$ 0.05 mm, internal diameter 1.15 $\pm$ 0.05 mm). The electrode was pulled on a vertical type electrode puller and the tip was broken back to 6-9 μm under microscopic control. Each barrel of the electrode contained a fine glass fibre along its length to facilitate the filling of the drug solutions to the tip by capillaric attraction.

Preparation of Drug Solutions

Drug solutions were prepared in clean detergent free glasswares, and with particle free distilled water or normal saline. Where necessary the pH of a given solution was adjusted with HCl or NaOH for optimum ionization. Table 1 gives the details of concentration and pH of the solutions used in these investigations. Drug solutions

are usually kept refrigerated until needed for use.

Filling the Microelectrodes

Filling of solutions into each barrel of the electrode was acheived by inserting a long small injection needle into each barrel as near to the tip as possible. Drug solution was injected from a small syringe at a slow rate to prevent formation of air bolus trapped in the barrel, which may cause insufficient filling. When glass tubings with glass fibre rib were used, the cappillary attraction created by the strand of glass fibre was usually effective for drawing the solution up to the tip.

Electrode Properties

After filling with drug solution, the electrode is then subjected to electrical tests. The most convenient and useful assessment is to measure the DC resistance of each barrel in the electrode assembly. In practice, the usual resistance range of 4 M NaCl filled barrel of a sevenbarrel microelectrode is 4-8 MΩ (Megohm), 8-12 MΩ For 2 M NaCl and 15-100 MΩ for barrel containing drug solutions.

Iontophoretic Ejection

Ejection of substances into the vicinity of neurones was achieved by passing current with the same polarity as that of the active ions species through the electrode barrels. For a strongly ionised ions the ejection depends mostly on iontophoretic migration, while in case of poorly ionised substance the release has to be dependent on electro-osmosis (see Curtis, 1964; Kelly et al, 1975).

This process could be enhanced by dissolving the particular substance in 165 mM NaCl. When being released the expelled sodium ions were believed to be accompanied with water and solute (i.e. drug) molecules. Substances released by this process are shown in Table 1.

Spontaneous Efflux and Retaining Current

To avoid spontaneous efflux of the contents inside the electrode (see Curtis, 1964; Kelly et al, 1975) a current with opposite polarity to that of the active ion species was continuosly passed through each barrel. The currents of 10-30 nA were usually used in these study. Theoretically, the amount of this retaining current was dependent upon the resistance of each particular barrel.

The Microiontophoretic Panel

The current pump or 'panel' used for generating electric current in this study was constant current electronic type, and was modified from the circuit of Gellar and Woodword (1972) by C.J. Courtice. This panel also incorporated an automatic current balancing channel. At all time this channel automatically provided the current which was equivalent to algebraic sum of the current flow at the electrode tip but of opposite polarity through one barrel which contained either 2 M NaCl or 2 M sodium acetate. By so doing, the potential at the tip of the microelectrode would remain zero throughout. This in turn reduced possibility of current flow per se influencing neuronal firing.

Table 1. Substances used in microiontophoretic study.

Substance	Abbreviation	Conc.(M)	pH	Supplier
Angiotensin II	A II	0.001	4.5	Calbiochem
Sar^1-Ala^8-angiotensin (saralasin)		0.001	4.5	Calbiochem
ɣ -amino-butyric acid	GABA	0.2	3.5	Sigma
Glycine HCl	GLY	0.2	3.5	Sigma
Taurine	TAU	0.2	3.5	Fluka AG, chem.
Glutamate, monosod	GLU	0.5	7.5	Sigma
L-aspartate	ASP	0.5	7.5	Sigma
Noradrenaline HCl	NA	0.2	4	Sigma
5-hydroxytryptamine Creatinine-sulphate	5-HT	0.2	4	Sigma
Bicuculline metho - chloride	BMC	0.005*	3.5	semisynthesised

* prepared in 165 mM sodium chloride solution

Electrophysiological Technique and Processing of Electrophysiological Data

Recording electrodes

Extracellular unit activity was recorded by using 4 M NaCl filled centre barrel of a seven-barrel microelectrode (the outer barrels was used for microiontophoresis application of drugs and balancing current).

Amplification and display of unit activity

To minimize electromagnetic and electrostatic interferences sucessful recording was performed on the animal housed in an earthed screened cage (Faraday cage). Figure 4 shows detail of experimental arrangement used in all electrophysiological investigation in this study. Two stages of amplification were employed. Unit activity was dilivered as single ended input into a high impedance probe (not show in Figure 4) which functioned as a unity gain buffer amplifier. The probe situated close to the recording electrode to minimize the "stray" capactive effect. Output from the high impedance probe was fed into a microelectrode amplifier (usually x 500, Nihon-kodhen) which has filter system to minimize both low frequency interference associated with line frequency and animal pulse and breathing movement and also high frequency interference from radio-transmission and switching artifact. The final output from an amplifier were displayed on a digital memory oscilloscope (Nihon-kodhen, VC 10).

Processing of spike data

In order to obtain signals which were compatible with the input stage of the instruments, the amplified spike potentials were

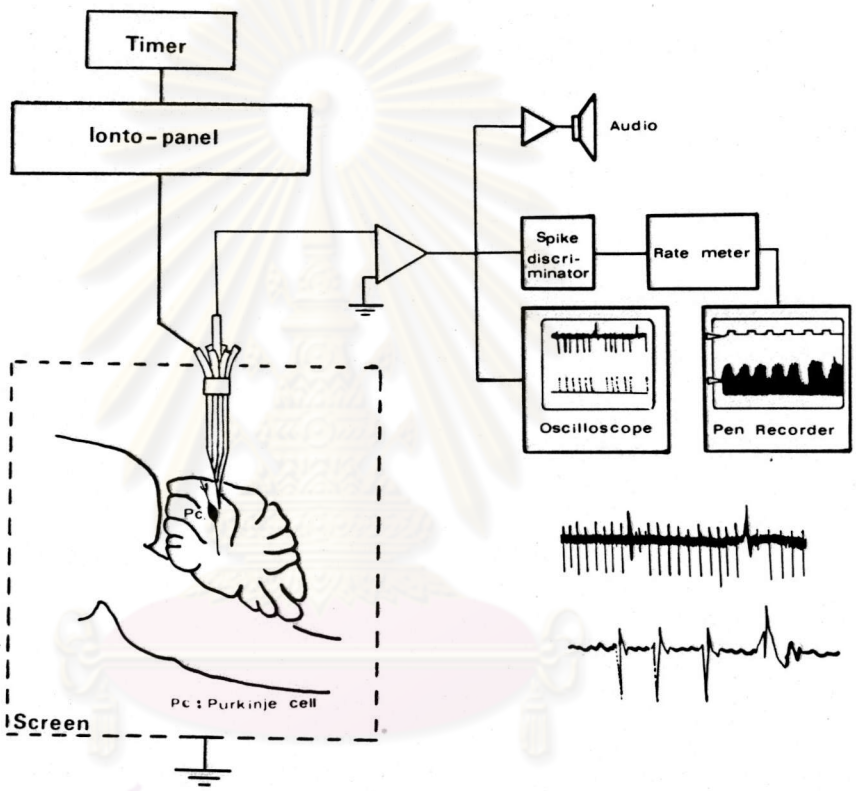

Figure 4. Diagramatic representation of experimental arrangements routinely employed in experiments involving electrophysiological investigation and microiontophoresis. For detail, see text.

converted into corresponding pulses of standard amplitude and duration. This was achieved firstly by feeding the amplified spike in to a pulse height selector, which by adjusting a variable gate, would reject undesirable signals (i.e. noise) which had amplitudes below or above a set level. This method together with manoeuvering the electrode position in relation to a particular neurone enabled a single unit spike activity to be isolated. The selected amplified spikes were then used for tiggering standard pulses (one pulse per spike).

The standard pulses were fed into a digital rate meter and epoch counter, the output from epoch counter was recored on a strip chart recorder as histogram of number of pulses over each consecutive epoch of 0.5 second. The record were subsequently converted to neuronal firing rates of spikes per second for data presentation. The neuronal activities bofore, during and after iontophoretic applications of various substances were compared and analysed.

The Criteria Used to Indentify Purkinje Cells in Cerebellum

On passing the microelectrode into the cerebellar cortex, the microelectrode encountered an increased level of maintained multiunit activity. Of the units that could be isolated in or near the Purkinje layer, most generated two distinctly different kinds of spike (Figure 5).

A simple spike-so-called because of its relatively simple waveform-was first recorded as an initially negative potential that commonly became positive-negative on further advance of the microelectrode and fired (without indications of injury) at maintained frequencies about 20 - 150 spikes/sec. Discharge of this simple

spike was frequently interrupted by another waveform that was more complex in shape (Figure 5, black dot). The "complex" spike occured in a sporadic pattern at a frequency about 1 - 4 spikes/sec, and was followed by a brief silent period. Units whose activity was recorded extracellularly in the cerebellar cortex and which generated these two different spike shapes are subsequently referred to as "Purkinje cells" (Eccles, 1973; Szentagothai and Arbib, 1974; Tebēcis, 1974; Thach, 1968).

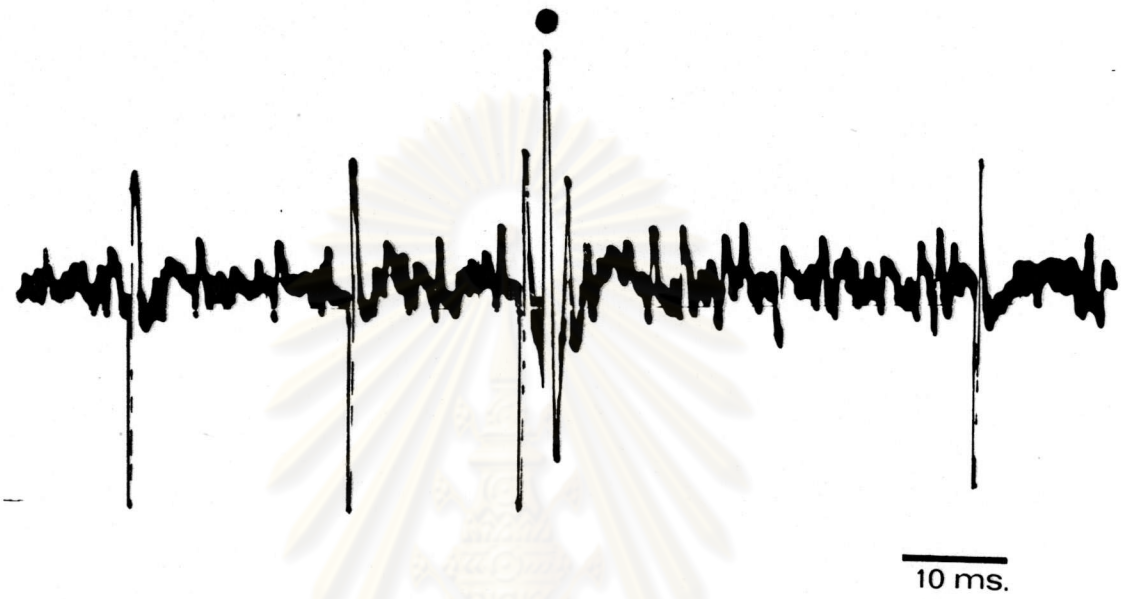

Figure 5. Maintained discharge of a Purkinje cell, recorded extracellularly, showing its two different spike potentials the "simple" and the "complex" (black dot). The traces shows their different pattern of discharge and shape. Positive is up.