Chapter II

General Method

Experimental Animal and Anaesthesia

All electrophysiological investigations were conducted on rats (male Wistar, weighing 190–300 g) anaesthetised with urethane. The animal received a single intraperitoneal injection of 1.4–1.7 $g.kg^{-1}$ urethane (ethylcarbamate).

Stereotaxic Techniques

In principle, electrode or cannulae are introduced into specific areas by referring to a three-dimentional system of co–ordinates determined by external landmarks on the skull or by other reference points from pre–existing atlas of the rat brain (Pellegrino, Pellegrino, and Cushman, 1979).

The animal was mounted on a stereotaxic apparatus (Narishige). 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 nape. The scalp was reflected to expose the skull, and attached muscles scraped away. The musculature covering the nape was cleared to expose the cisterna magna. To minimize cerebral edema and pulsation, a small incision was made through the cistern to provide a drainage for cerebrospinal fluid. The skull over the areas to be investigated was opened 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 warm normal saline.

A small patch was then made in the pia matter to facilitate the insertion of glass microelectrode. This step was considered necessary since advancing a microelectrode through an intact pia matter tended to cause blockage. After insertion of the electrode, the exposed areas of the brain were covered with warm 4% agar in normal saline to minimize pulsation which might interfere with electrophysiological observation.

Microiontophoretic Techniques

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 interchagable with

microelectrophoresis, but in a more strict sense the term microiontophoresis should be applied only when describing the movement of ions by currrent flow. Thus, in microelectrophoresis, microiontophoresis occurs, which usually is associated with electro–osmosis.

Microelectrode

In most cases, when microiontophoretic application of substances was performed in this study, five and seven–barrel microelectrodes were used. Microelectrode was fabricated in the laboratory from borosilicated glass tubing (external diameter 1.55 ± 0.05 mm, internal diameter 1.15 ± 0.05 mm). The electrode was pulled on a vertical type electrode puller and the tip was broken back to 6–9 microns 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 glassware, 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.

Dioscorine Extraction

Process for DCR extraction was modified from the method described by Pavovat (1973). DCR based was subsequently converted to hydrochloride salt to yield a white precipitate (salt preparation was by courtesy of Assistant Professor Dr. Chamnan Patarapanich, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

Filling the Microelectrodes

Filling of solutions into each barrel of the electrode was achieved by inserting a long small injecting 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 might cause insufficient filling. When glass tubings with glass fibre rib were used, the capillary attraction created by the strand of glass fibre was usually effective for drawing the solution up to the tip.

Table 1 Substances used in microiontophoretic study

Substance	Abbreviation	Conc.(M)	pH	Supplier
Dioscorine HCl	DCR	0.1	4.5	synthesised from DCR based which was extracted from kloi
γ–aminobutyric acid	GABA	0.2	3.5	Sigma
Glycine HCl	GLY	0.2	3.5	Sigma
Taurine	TAU	0.2	3.5	Sigma
Noradrenaline HCl	NA	0.2	4	Sigma
5–hydroxytryp–tamine HCl	5–HT	0.2	4	Sigma
L–glutamate monosodium salt	GLU	0.5	7.5	Sigma
L–aspartic acid	ASP	0.5	7.5	Sigma

Bicuculline methochloride	BMC	0.005*	3.5	Sigma
Strychnine sulfate	STRY	0.005*	7	Macfarlan Smith

*prepared in 165 mM sodium chloride solution

Electrode Properties

After filling with drug solution, the electrode was then subjected to electrical tests. The most convenient and useful assessment was to measure the D.C. resistance of each barrel in the electrode assembly. In practice, the usual resistance range from 4 M NaCl filled barrel of a five or seven barrel microelectrode was 4–8 MΩ (Megaohm), 8–12 MΩ for 2 M NaCl and 15–100 MΩ for barrel containing drug solution.

Iontophoretic Ejection

Ejection of substances into the vicinity of neurones was achieved by passing current with the same polarity as that of the ion species through

the electrode barrel. For a strongly ionized ions the ejection depends mostly on iontophoretic migration, while in case of poorly ionized substance the release has to be dependent on electro–osmosis (Curtis, 1964; Kelly, Simmonds, and Straughan, 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 dissolved solute (i.e drug) molecules. Substance released by this process are shown in Table 1, as denoted by using 165 mM NaCl solution as vehicle.

Spontaneous Efflux and Retaining Current

To aviod spontaneous efflux of the contents inside the electrode a current with opposite polarity to that of the active ion species was continuously 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 (Curtis, 1964, Kelly et al., 1975).

The Microiontophoretic Panel

The current pump used for generating electric currrent in this study was constant current electronic type. This device 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.

Electrophysiological Technique and Processing of Electrophysiological Data

Recording Electrodes

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

Amplification and Display of Unit Activity

To minimize electromagnetic and electrostatic interferences successful recording was performed on the animal housed in an earthed-screen cage (Faraday cage). Figure 4 shows detail of experimental arrangment used in all electrophysiological investigation in this study. Two stages of amplification were amployed. Unit activity was delivered as single ended input into a high impedance probe (not show in Figure 4)

which funtioned as a unity gain buffer amplifer. 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×500, Nihon–Kodhen) which had filter system to minimize both low frequency interference associated with line frequency and animal pulses and breathing movements, and also high frequency interference from radio–transmission and switching artifact. The final output from the amplifier was 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 potentails were converted into corresponding pulses of standard amplitude and duration. This was achieved firstly by feeding the amplified spike into a pulse height selector, which, by adjusting a variable gate, would reject undersirable signals (i.e. noise) which had amplitudes below or above a set level. This method together with manoeuvreing 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 triggering 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 record on a McIntosh

Computer (LC 630) emulated as a pen recorder with a digital–to–analog converter (MacLab) and a software (chart V.3.4.2, MacLab), to obtain the neuronal firing rates and the counts displayed as peristimulus–time histogram of number of pulse over each consecutive epoch of 0.5 sec. The record were subsequently converted to neuronal firing rates of spikes per second for data presentation. The neuronal activities before, during and after iontophoretic applications of various substances were compared and analysed.

The Criteria Used to Identify Purkinje Cells in Cerebellum

Study of the effects of substances under investigator was made on cerebellar Purkinje cell, owing to the fact that this cell possesed variety of receptors corresponding to a number of putative neurotransmitters,namely, GABA, glycine, taurine, noradrenaline,5–HT, glutamate, and aspartate. On passing the microelectrode into the cerebellar cortex, the microelectrode encountered an increased level of maintained unit activity. At the depth of Purkinje cell layer, typical unit with two different kinds of spike, the complex and simple spikes, was identified as those generated by a Purkinje cell (Figure 5).

A simple spike, so–called because of its relatively simple wave form, 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 wave form 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 cell" (Eccles, 1973, Szentagothai and Arbib, 1974, Tebecis, 1974).

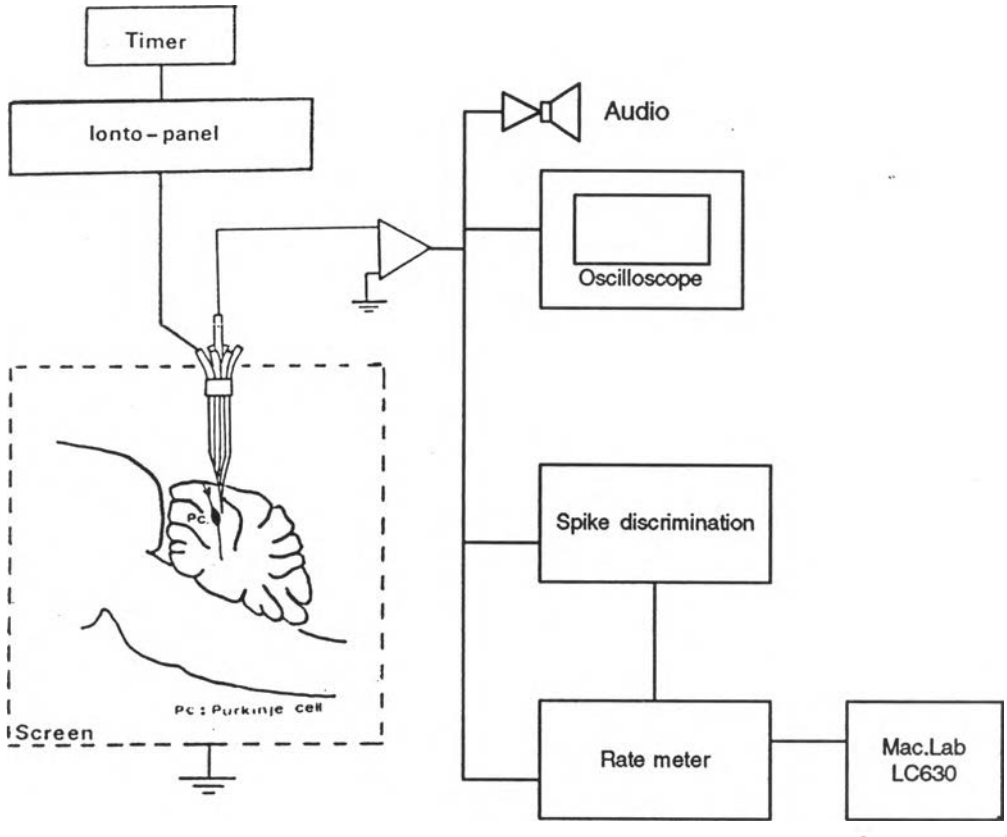

Figure 4 Diagramatic representation of experimental arrangments routinely employed in experiments involving electrophysiological investigation and microiontophoresis. For detail, see text

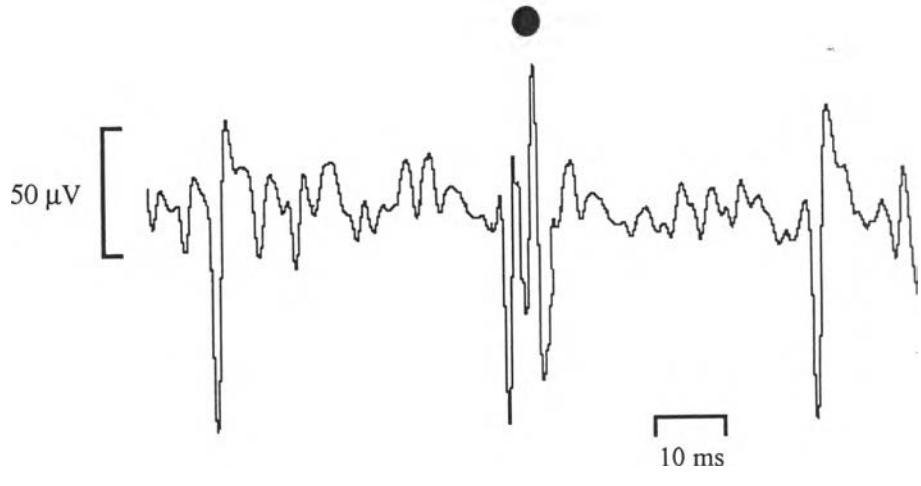

Figure 5 Maintained discharge of a Purkinje cell, recorded extracellularly, showing its two different spike potentials the "simple" and the "complex" (black dot). The traces show their different pattern of discharge and shape, positive is up. This tracing was transferred from a digitally stored data on a storage oscilloscope.