DIRECT EXTRACELLULAR APPLICATION OF DRUGS

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NEUROPHARMACOLOGICAL investigations can be subdivided into several kinds depending upon the type of information which is being sought. For some purposes it is sufficient to administer a drug to an intact animal and to observe behavioural changes and responses to predetermined alterations in the environment. A more sophisticated approach makes use of the reflex responses of particular portions of the central nervous system—thus the action of drugs on respiratory, cardiac, visceral or muscle reflexes can be studied. For these types of investigations drugs can be administered orally, intramuscularly, intravenously, intra-arterially or can be applied topically to the surface of nervous tissue. However, these techniques rarely permit the actual site and mode of action of a particular chemical compound to be determined.

Over the past decade it has become possible to study individual neurons within various portions of the central nervous system by means of microelectrodes. The electrical correlates of excitation and inhibition can be described in fairly specific terms (Eccles, 1957), and detailed analyses of these phenomena have led to the establishment of the chemical theory of synaptic transmission. It was considered necessary that drug effects should also be studied at the cellular level, not only to determine the mode and site of action of pharmacological agents, but also to establish the nature of the chemical synaptic transmitter substances. If the behaviour of a single cell is observed with a microelectrode and a drug is administered systemically or applied topically to the tissue, two major factors may complicate the interpretation of subsequent observations. In the first place diffusional barriers may prevent the access of the added material to neurons. There is good evidence for a blood-brain barrier towards certain compounds such as curare, 5-hydroxytryptamine, prostigmin, and bacterial toxins (cf. Curtis and Eccles, 1958b) and in addition there may be diffusional barriers more intimately associated with synapses upon neurons. Secondly, in view of the widespread nature of the drug application, and the complex synaptic interconnexions between cells throughout the central nervous system, it becomes relatively difficult to ascribe an action—either excitation or

depression—to a direct effect upon the particular cell being observed. Many substances are also powerful activators of peripheral sensory receptors (Gray, 1959), and impulses so produced may modify neuronal activity. Other factors which have to be considered, when interpreting results obtained from systematic application of drugs, include the possible production of generalized or localized vascular changes, the possibility that the added agent may be rapidly inactivated enzymically, thus lowering the effective circulating concentration, and the limitation which may have to be placed upon dosage because of more generalized effects upon the experimental animal.

For these reasons techniques have been developed whereby drugs and possible transmitter can be tested for their actions upon single nerve cells by applying them directly into the extracellular environment of the cell. The methods used are based upon those of Nastuk (1953), the substances being passed from fine glass micropipettes by the process of electrophoresis (Curtis and Eccles, 1958a; Curtis, Phillis and Watkins, 1959, 1960). From a knowledge of the dissociation constants of a particular compound and its stability, the pH of an aqueous solution is altered until there is a predominance of a charged over the uncharged form of the substance (cf. Curtis and Watkins, 1960). The solutions are made up to have maximal conductance and are usually saturated. Diffusional loss from the tip is controlled by an electrical current applied between the top end of the electrode and the mass of nervous tissue. Active cation or anion can be applied by reversing this current. Many experiments have established that cations such as K^+ and Na^+ and anions such as Br^-, I^-, Cl^-, SO_4^{2-}, PO_4^{3-}, bitartrate, and *p*-toluenesulphonate are inert when applied extracellularly in this fashion. Consequently for most pharmacological agents a suitable salt can be chosen in which one ion species is inactive. A lower limitation of 3 has to be placed upon the pH of solutions since hydrogen ion itself has an excitant action on neurons (Curtis, Phillis and Watkins, 1961). Since the orifices of the electrodes are of the order of 1–10μ, two processes are involved in the ejection of material from the tip—ionophoresis and electro-osmosis. In the first, ions are applied by current flow, the rate of application being related to the magnitude of the current. With electro-osmosis there is a flow of solvent depending upon the presence of an electrical double-layer at the glass-solvent interface (cf. Curtis, Perrin and Watkins, 1960). With the type of glass used in these investigations electro-osmosis favours the transfer of cations from the electrode and the relationship between current and rate of ejection is complex. Thus the quantitative aspects of this method of drug application are difficult to control. However, even if the rate of ejection was known, the extracellular space of the central nervous system is such a complicated structure (Horstmann and Meves, 1959) that calculations of the concen-

tration attained near neurones are impossible. Nevertheless, it is possible to obtain some idea of the concentrations attained and consequently a rough quantitative comparison can be made of different compounds, particularly when a multi-barrel electrode assembly is used to apply several compounds to a single cell from approximately the same place.

The action of drugs applied extracellularly have been determined upon neurons in various portions of the nervous system (Curtis, 1961a). In order to identify and classify the individual cells it is necessary to be able to excite or inhibit them along selected afferent pathways or to excite their axons. Occasionally the site of recording requires to be marked by passing metallic complexes from the recording microelectrode

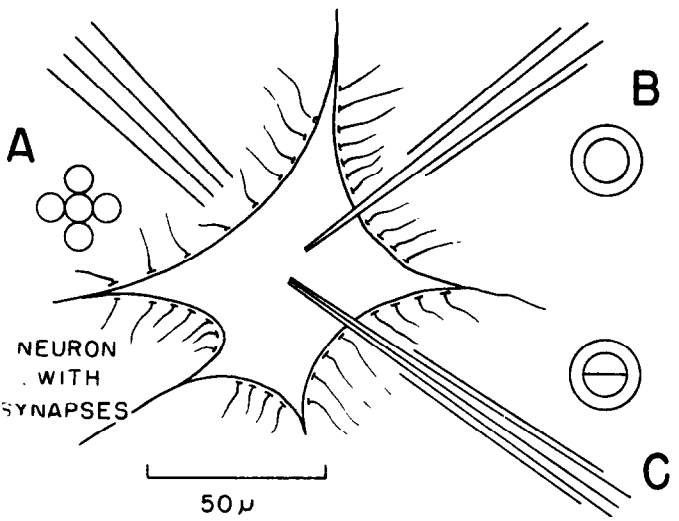

FIG. 1. Diagrammatic scale drawing of the types of microelectrodes used for applying drugs to neurons, together with their cross-sections. A, five-barrel electrode; B, single-barrel co-axial electrode; C, double-barrel co-axial electrode.

so that subsequently the area can be identified histologically (Galifret and Szabo, 1960). Although the recording of extracellular responses from groups of cells may be adequate to determine whether or not a particular substance has an action upon neuronal membrane, the interpretation of alterations in field potentials generated by the activity of many neurons may be difficult (Curtis *et al.* 1959), and it is essential to study the action of the drug upon single neurons. Extracellular records of spike potentials (0·5–1 mV) are obtained with relatively large electrodes, the recording electrode being fused with either one drug-containing barrel (double electrode) or with four—as in the five barrel electrode illustrated in Fig. 1A. The advantage of this latter electrode is that as the applications are made at a similar distance from the cell,

the concentrations attained by the different compounds will be proportional to the electrophoretic current used, thus permitting relative potencies to be determined. In addition a more complete study of synaptic transmission at a particular neuron can be carried out by applying substances which mimic, block or potentiate the action of the natural transmitter.

In general, an extracellular electrode merely records the presence or absence of spike potentials. Consequently drug application in association with extracellular recording determines whether an agent excites or depresses a cell but does not permit a close analysis of how this is done. Various tests can be applied, as will be discussed below, but a detailed analysis of drug action necessitates the combination of intracellular recording with extracellular drug application. The use of an intracellular electrode, measuring the potential across the membrane of the cell, allows changes in this potential to be observed, alterations in the magnitude of post synaptic potentials to be measured and both the direct excitability and conductance of the membrane to be determined. Figure 1 illustrates the types of co-axial electrodes which have been used. The central recording barrel, either single (B) or double (C) with a tip of 0·5–1μ, projects beyond the outer barrel which remains extracellular. The double-barrel intracellular electrode permits the alteration of the membrane potential so that changes in individual ionic conductances can be estimated (Eccles, 1957).

The particular advantage of the electrophoretic technique of drug application is the ability to apply substances at close range to neuronal receptors, thus enabling the chemical sensitivity of these membrane sites to be determined. Most of the disadvantages derive from an inadequate knowledge of drug concentration, together with factors associated with the use of current for applying chemical agents. Since synapses occur diffusely over the surface of neurons it is reasonable to expect that chemical compounds applied to the extracellular space diffuse to some of the synaptic areas. The assumption that the concentrations attained are adequate to be of pharmacological significance can only be justified when the results are comparable with those determined by more conventional methods of administration. However these latter methods, as discussed earlier, have their own complications and difficulty arises when results obtained electrophoretically differ consistently from those obtained by other means (Curtis and Koizumi, 1961; Curtis *et al.* 1961). If it can be shown that a particular substance is being ejected from a micropipette at a suitable rate, and if other substances applied from different barrels of the same electrode produce results in accordance with those observed using other methods of application, then results obtained electrophoretically can be accepted in preference to those obtained by other methods.

The necessity of using an electrical current to apply chemical compounds to neurons occasionally produces difficulties, since neurons may be influenced by current flow. Simple tests can be applied to exclude this complicating factor, which rarely arises if care is taken to restrict investigation to those cells producing negative-positive extracellular spike potentials of 0·5–1 mV. in amplitude and to limit the electrophoretic currents to 100–200 $\times 10^{-9}$ A. In general, current effects coincide in time course with the current flow whereas drug effects have latencies of at least 50 msec and may persist for several seconds after the current is terminated. Usually if current in one direction excites a neuron, a current in the reverse direction depresses. Further, as neither Na^+ nor Cl^- ions effect neurons when applied electrophoretically, the sodium chloride-filled recording barrel serves as a control since responses evoked by passing current through it can only be due to current flow (cf. Curtis and Koizumi, 1961).

This brief account of the electrophoretic method of investigating drug action can be concluded by referring to several recent investigations which illustrate the methods adopted to determine the mode ofaction of compounds. The failure of γ-amino-*n*-butyric acid to effect the membrane potential of motoneurones excluded this substance as an inhibitory transmitter acting upon these cells (Curtis *et al.* 1959). A large series of related amino acids have been investigated for their action upon spinal neurones (Curtis and Watkins, 1960), a smaller series being tested upon neurones in the brain stem and lateral geniculate nucleus. Of considerable interest was the observation that some of the acidic amino acids were excitants of neurones in all of these areas. This excitation was considered not to be related to excitatory synaptic transmission (Curtis, Phillis and Watkins, 1960), but recent experiments upon motoneurones have established this beyond reasonable doubt. Although many criteria must be established before a compound can be definitely labelled as a transmitter at a particular neurone (Paton, 1958; Curtis, 1961b), it is absolutely essential that its local application causes the presumed sub-synaptic site of action to exhibit changes identical with those evoked by the synaptic transmitter. The synaptic excitation of motoneurones is associated with a depolarization or excitatory post-synaptic potential (EPSP) due to an increase in the permeability of certain regions of the membrane to Na^+, K^+ and possibly also to Cl^- ions. These ionic movements are in equilibrium at a membrane potential near zero (Coombs *et al.*, 1955) and for a substance to be considered an excitatory transmitter of motoneurones, its local application to these cells must evoke a depolarization having an equilibrium potential identical with that of the EPSP. Under these conditions the conductance change produced by the compound would be almost certainly identical with that of the transmitter. Recent experiments using double barrel

co-axial electrodes have shown that the equilibrium potential for the depolarization produced by several potent acidic amino acids is at a lower level of depolarization than the equilibrium potential for synaptic excitation. This experiment was performed by determining the magnitudes of an EPSP and a depolarization evoked by constant amounts of the amino acid at different levels of membrane potential, these levels being maintained by passing current through one barrel of the intracellular double electrode. The amino acids failed to depolarize at a membrane potential approximately 20–30 mV positive to the resting level, and at potentials even more depolarized than this, evoked a hyperpolarization. At this level of resting potential however, the EPSP was still a depolarization and it is therefore highly improbable that the amino acids are naturally occurring excitatory transmitters upon these neurons.

Caution is occasionally necessary when attempting to interpret results which suggest that a compound is an excitatory transmitter. It has been found that some neurons in the brain stem and lateral geniculate nucleus can be excited by acetylcholine. In some cases this excitation was prevented by dihydro-β-erythroidine (DHβE), in others DHβE was without effect. However in all cases DHβE did not block the synaptically evoked and spontaneous spike responses (Curtis and Koizumi, 1961; Curtis and Davis—unpublished observations). As DHβE is an extremely potent blocking agent of Renshaw cells, the only mammalian neurones for which acetylcholine has been shown to be an excitant transmitter (Eccles *et al.*, 1954; Curtis and Eccles, 1958a), it is reasonable to assume that if the orthodromic activation of a neuron, and this will include spontaneous activity due to random synaptic bombardment, is indeed cholinergic, such excitation should be diminished by DHβE. Thus it may not be sufficient merely to demonstrate the sensitivity of a neuron to choline esters. In addition, the use of specific blocking agents and enzyme inhibitors may be necessary to establish fully the cholinergic nature of excitatory synaptic transmission. It is possible that the apparent excitatory action of acetylcholine is due to an action upon acetylcholine-like receptors.

The final investigation to be discussed illustrates methods by which the mode of action of compounds can be investigated without the necessity of intracellular recording. The use of intracellular electrodes in certain regions of the central nervous system is rendered difficult by the size of the neurones and the presence of respiratory and vascular pulsations. However sufficient information may be gained from extracellular recording provided multibarrel electrodes are used and various means of stimulating neurons are employed. Thus an excitant amino acid, such as L-glutamic acid, can be applied to test the excitability of a neuron, the orthodromic excitatory responses of which have been blocked by a depressant drug. Since amino acid excitation is not

associated with synaptic mechanisms there will be no change in the frequency of firing produced by L-glutamic acid if the depressant merely blocks the post-synaptic action of the excitatory transmitter (cf. Curtis, Phillis and Watkins, 1960). On the other hand if synaptic excitatory action is reduced because the depressant alters the neuronal membrane conductance, the excitant amino acid will also be less effective (Curtis and Watkins, 1960). Another method of testing the excitability of post-synaptic neuronal membrane consists of making observations upon the antidromic invasion of the neuron by impulses initiated in the axon of the cell. The blockage of antidromic invasion by a drug may indicate that it affects the membrane conductance of the neuron. On the other hand the failure of a depressant of synaptic excitation to alter antidromic invasion indicates that no such conductance change is produced.

A recent investigation on neurons in the lateral geniculate nucleus of the anaesthetized cat serves to illustrate these techniques (Curtis and Davis, 1961). These cells can be excited monosynaptically by impulse in the optic nerve and the excitation is depressed by intracarotid injections of D-lysergic acid diethylamide or certain tryptamine derivatives (Evarts, 1956, 1958; Bishop *et al.*, 1961). 5-Hydroxytryptamine (5-HT) failed to influence these neurons when administered by intra-arterial injection, presumably because of the blood-brain barrier. 5-HT has been considered a possible central transmitter substance and as D-lysergic acid diethylamide and related compounds block the excitatory action of 5-HT upon smooth muscle, it was of great interest to apply 5-HT to geniculate neurons. When applied electrophoretically as a cation, 5-HT readily blocked the orthodromic excitation of geniculate neurons. Several conclusions could be drawn from this observation but the inference that the indole was an inhibitory transmitter was rendered impossible by its failure to influence both the antidromic invasion of the neurons by impulses initiated in the optic radiation and the firing of the neurons evoked by an applications of L-glutamic acid. Thus it can be assumed that 5-HT either prevents the release of the excitatory transmitter from optic nerve terminals or blocks its access to post-synaptic receptors.

In conclusion it can be stated that despite certain disadvantages the local electrophoretic application of substances to neurons is an invaluable technique for neuropharmacological studies. Many observations can be made using different neurons in the one animal and the possibility that a compound is a transmitter can be explored by applying it close to its presumed site of action. In this way factors which complicate a more generalized administration are overcome and the use of specific blocking agents and enzyme inhibitors allow of a comparison between the pharmocology of the suspected and the natural transmitter.

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