

INVESTIGATION OF BRAINSTEM
TRANSMITTER SYSTEMS

Pharmacological Studies in the Red Nucleus of the Cat

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ERRATUM.

In an introductory note it is stated that a paper has been presented for publication entitled ' Effect of 5-hydroxytryptamine, noradrenaline and dopamine on red nucleus neurones '. The correct title is ' The action of 5-hydroxytryptamine and noradrenaline on red nucleus neurones '. This paper is to be presented to the 7th meeting of the Australian Physiological Society, February 1965.

NOTE

A portion of the results included in this thesis have been presented for publication as a preliminary report to the editors of 'Experientia', Basel¹.

It is intended to present the results, in their entirety, to another journal, probably the Journal of Neurophysiology, at a later date.

1. 'Effect of 5-hydroxytryptamine, noradrenaline and dopamine on red nucleus neurones'. R. Davis, P.C. Vaughan and M.J. Rowe.

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General Introduction

The red nucleus is a part of the so-called 'extrapyramidal system' (Brodal, 1963) and to lesions of this nucleus have been attributed dyskinetic phenomena clinically observed in man (see Carpenter, 1961).

Catecholamines, especially noradrenaline, have been found in grey matter of the brainstem, while dopamine is strongly localized to the basal ganglia and structures related to them anatomically and in their control of motor function. The basal ganglia, comprised of the caudate nucleus, putamen, globus pallidus, internal capsule and claustrum are a part of the 'extrapyramidal system' and evidence of disordered dopamine metabolism in basal ganglia disease (Barbeau and Sourkes, 1961) implicates this compound in their function. The observation that dopamine is most prominent in the phylogenetically youngest structures (Bertler, 1961) would also seem to contradict the possibility of the presence of this amine being atavistic.

The object of the experiments entailed in this thesis was to apply biogenic amines and some related compounds to red nucleus cells, micro-electrophoretically, with a view to attempting to elucidate whether or not those amines which are found in this nucleus are concerned in synaptic function.

Chapters One and Two constitute a review of relevant literature.

Chapter One deals with the growth of the concept of the synapse and some recent observations on junctional morphology, as well as an outline of mechanisms subtending the phenomenon of synaptic transmission. A knowledge of the facts discussed in this chapter is essential before an attempt can be made to study the pharmacology of central synaptic transmission and it serves as a prolegomena for the rest of the treatise.

As will be evident in the results, catechol- and indoleamines are apparently more intimately related to synaptic processes in the red nucleus than acetylcholine. A brief review of histo- and neurochemical observations on catechol- and indoleamine distribution and localization in the central nervous system is included in Chapter Two as is a review of the pharmacological work which has been carried out on the central nervous system, using the method of microapplication of drugs immediately into the environment of neurones, using the electrophoretic technique.

As an immediate preface to the experimental section a brief description of the anatomo-physiological context of the red nucleus is given in order to orient the reader to the structure and function of this part of the brainstem.

CHAPTER I

THE SYNAPTIC MECHANISM

Concepts of Chemical Transmission, Studies of Transmitter
Release and Morphology of the Junctions

The development, over the last decade or so, of sophisticated electrophysiological apparatus and the increasing use of the electron microscope have heralded in an era of unprecedented expansion in research on and understanding of biological mechanisms, not the least significant of these being the mechanism of information conduction in nerve fibres and transmission at neuronal and neuromuscular junctions.

In 1781 Fontana (cited by Grundfest, 1957), fired by the enthusiasm for explanations of all 'vital' phenomena in terms of 'electricity' which gripped the entire scientific world following the discovery of the Leyden jar, postulated that muscles may in some way be caused to contract, by some principle which, "if it not be the common electricity, may be something, however, very analogous to it ... The nerves should be the organs destined to conduct the fluid, and perhaps also to excite it". After this initial interest in its possible mechanism neuromuscular transmission was taken for granted as researchers, led by du Bois-Reymond and Helmholtz, devoted themselves to the formulation of theories of biological electrogenesis, the form of the nerve or muscle impulse and its propagation.

The time eventually arose when histological and electrophysiological experiments had provided sufficient data for the proposal of an electrical theory of junctional transmission. A theory that "a nerve only throws a muscle into contraction by means of its currents of action" was propounded (Kühne, 1888) and met with remarkably acute criticism by du Bois-Reymond, who was guarded, if not to be described as sceptical as to the validity of the so-called "discharge hypothesis". It was known at that time that a muscle responded with almost no latency to direct stimuli, whereas there was always an irreducible and appreciable latency

to indirect (nerve) stimulation: du Bois-Reymond remarked that only two alternatives are conceivable, secretion of an excitant substance from the nerve endings, or electrical excitation. Carl Sachs, a student of du Bois-Reymond, succeeded in stimulating a single nerve fibre, probably the first time this had been done, and observed the twitch of but the single innervated muscle fibre. This was condemning to the hypothesis that current flow from an excited end-plate (at that time still thought to be extrinsic to the contractile fibres) led to the discharge and contraction of surrounding muscle fibres: du Bois-Reymond was prepared to concede that with a special orientation of the nerve to the muscle transmission by electrical means may be possible. We now know that this orientation does not exist and that the leakage of current in the low impedance extracellular space precludes the possibility of stimulation of the muscle by an electrical coupling with the prejunctional nerve ending.

Claude Bernard's discovery of neuromuscular blockade by curare provided the first real stumbling block to the electrical theory of transmission, but adherents to the latter modified their concepts so as to account for pharmacological observations and it is only within recent years that some of these theories have finally been proven untenable. As a result of his labours, Lucas wrote in 1907 that "three excitable substances only have been excited and the identification of these as one muscle-substance, one nerve-substance and a substance lying about the region of nerve endings follows easily from the experiments made on muscle fibre alone, on nerve trunk alone, and from the action of curare". The next couple of decades saw the findings of Loewi, Dale, their contemporary greats and ardent disciples - information as was not forthcoming from the older physiological works - which are now basic and fundamental to students of pharmacology, but which in those days were laying the

foundations for the acceptance of the theory of chemical transmission. As a result of these works, acetylcholine was recognized as the transmitter at the neuromuscular junction (see Dale et al., 1936).

In 1952 Fatt and Katz observed spontaneous electrical activity at the motor end-plates of frog's sartorii, consisting of a random succession of "miniature end-plate potentials", the amplitude of which were of the order of one hundredth of the normal end-plate response to a motor nerve impulse (Fatt and Katz, 1951). Consistent with the theory of cholinergic production of post-junctional changes (see next section), these miniature potentials (m.e.p.ps) were greatly reduced in size by a small dose of curarine and increased in amplitude and duration by prostigmine. Similar findings were made at the end-plate in mammalian muscle (Boyd and Martin, 1956 a, b). A general observation on all neuromuscular junctions has been that the amplitude of the end-plate potential (e.p.p.) in response to nerve stimulation is affected by the external calcium concentration. Studies on this phenomenon have indicated that the amount of acetylcholine liberated by a motor nerve volley is a function of the relative amounts of calcium and magnesium ions present (del Castillo and Engbaek, 1954), these two ions being competitors for some presynaptic site (Hubbard, 1961) involved in the 'electrical' release of transmitter substance, an increase in calcium concentration tending to increase transmitter output. Reduction of the extracellular calcium concentration has been observed to decrease the size of the end-plate potential in a "step-wise" manner, involving an all-or-none blockage of a variable number of miniature components. In amphibia (Fatt and Katz, 1952) the size and frequency of the individual miniature potentials was not affected by calcium lack: in the mammal however, the frequency of the m.e.p.p. was determined by the external calcium - magnesium concentration ratio

(Boyd and Martin, 1956 b) and investigations on the isolated rat diaphragm preparation brought conclusions (Hubbard, 1961) that in the mammal at least, two release mechanisms may be involved. Total calcium deprivation of the solution bathing the rat diaphragm preparation left a background m.e.p.p. frequency which was unaffected by magnesium, whereas introduction of calcium to the extracellular medium brought the competitive mechanism to light. It would thus appear that calcium-dependent and calcium-independent mechanism may exist, the former not being readily demonstrable in amphibia. The calcium-independent mechanism exhibits a sensitivity to temperature and external osmotic pressure (Fatt and Katz, 1952; Hubbard, 1961) and appears to be a random process uncoupled to electrical events, whereas the calcium-linked component appears also to be voltage dependent (Hubbard, 1961). Similar miniature postsynaptic potentials occur in motoneurons and an analysis by Katz and Miledi (1963) indicates that the mechanism here is probably similar to that at the end-plate.

Prompted by a discrepancy which had been observed between the effect of hyperpolarizing currents upon the release of transmitter spontaneously and by nerve-impulses, Hubbard and Willis (1962) carried out an investigation on the effects of hyperpolarization of mammalian motor nerve terminals on the parameters of transmitter release. End-plate potentials elicited at two to four second intervals during the passage of hyperpolarizing currents increased progressively in amplitude to a plateau level (see Hubbard and Willis, 1962 b, figs. 3 and 4) which was sufficient to generate action potentials (see next section). This effect was manifest after a delay from the turning on of the current, this delay sometimes lasting several seconds and after the current was turned off, the e.p.p. amplitude fell, at first rapidly and then more slowly, often

taking many seconds to return to control height. The amplitude of m.e.p.ps was unaltered during hyperpolarizing currents despite concomitant e.p.p. increases and although frequency increased in normal Krebs-Ringer solution, it was unaltered when the magnesium concentration was raised to 11mM. Since hyperpolarization of electrically excitable tissue (see next section) increases the 'overshoot' of an invading action potential, due to 'activation' of the sodium conductance mechanism (Hodgkin and Huxley, 1952) it was thought (del Castillo and Katz, 1954 a) that the amount of transmitter released by a nerve volley may be related to the amount of 'overshoot' occurring in the action potential: this would be in partial agreement, at least, with the observation that presynaptic depolarization reduces the amount of transmitter released by a nerve volley (Hubbard and Willis, 1962 a) since prolonged 'conditioning' depolarization 'inactivates' the sodium conductance mechanism and spike overshoot is reduced or abolished. However, the long latency of onset of the effect of hyperpolarization on e.p.p. amplitude and the slow, progressive rise in amplitude during the passage of current (Hubbard and Willis, 1962 b) is not entirely accounted for by this interpretation, nor is the observed slow decline to control level after switching the polarizing current off. An alternative, or additive interpretation is that the availability of transmitter is dependent on resting membrane potential. This has been borne out in more recent experiments (Hubbard and Schmidt, 1963) in which extracellular recording with microelectrodes situated very close to preterminal endings has been employed. During and after repetitive stimulation of the nerve fibre innervating an isolated rat diaphragm the spike potential increased, this increase having a similar time course to the simultaneously recorded increase of e.p.p. amplitude, and the increased e.p.p. amplitudes were logarithmically proportional to the simultaneously increased spike amplitudes. However, after a single conditioning impulse, the e.p.p. response

to a second impulse at a close interval was potentiated, but the amplitude of the accompanying spike potential was decreased. The conclusions that can be drawn from these results are (i) the nerve impulses actively invade nerve terminals (ii) after repetitive stimulation the potentiation of e.p.p. amplitudes is due to an increase in presynaptic spike amplitudes induced by hyperpolarization of nerve terminals and (iii) after single impulses the potentiation of e.p.ps is brought about by another mechanism, possibly an increased availability of acetylcholine. Of course presynaptic depolarization will result in the converse of these effects: this is the basis for the explanation of "presynaptic inhibition" (see next chapter). The observation that m.e.p.p. frequency is not increased by hyperpolarization cannot be satisfactorily explained unless it be assumed that m.e.p.p. frequency is not closely linked to the amount of available transmitter.

Morphology of the Junction: the Subcellular Units

It has been pointed out (Eccles, 1964) that the first published concept of the structure of the central nervous system was by Gerlach in 1871. He pictured it as a net-like structure, or reticulum, the nerve cells themselves being at the junctions, or nodes of the ramified anastomosed fibres. This concept was challenged shortly afterwards by His and Forel who both conceptualized nerve cells as being independent units which were contiguous, but not continuous, with one another: this was the beginning of the neurone theory. Good histological support for the neurone theory was provided by the work of Cajal, which was done around the turn of the century using the staining technique of Golgi, but it was many years before the question was finally settled in favour of this doctrine.

Investigations with light microscopy (Bodian, see Palay, 1958) indicated the separation of pre- and postsynaptic fibres by a thin membrane, the synaptolemma, but the electron microscope has shown this to be subdivisible into two apparently bilaminar membranes interrupted by a cleft approximately $200\text{\AA}$ wide ($\text{\AA} = \text{Angstrom unit} = 10^{-10}$ metre) in the neuronal synapse (Palay, 1958). These membranes and the 'synaptic cleft', which is continuous with the extracellular space, assert the individuality of the nerve cells and are final proof of the validity of the neurone doctrine. In an extensive study of the morphology of synapses in several region of the rat brain (Palay, 1958) simple nerve endings have been demonstrated as small, circumscribed, rounded expansions abutting upon the surfaces of perikarya (nerve cell bodies) and dendrites: these terminals may be so closely packed (for example, as they appear in the rat medulla) that they may be arranged next to one another like palisades surrounding, and almost encapsulating, the neurone. The nerve endings in the central nervous system contain two characteristic components: a cluster of mitochondria, and a throng of small vesicles, which are apparently secretory inclusions. Techniques for isolating these vesicles have been described by several workers (e.g. de Robertis et al., 1962; de Robertis et al., 1963; Whittaker et al., 1963; Michaelson and Whittaker, 1963; Maynert et al., 1964) whose results have shown the vesicles to be of remarkably uniform size distribution and to contain compounds (e.g. acetylcholine) which are assumed to play a role in synaptic transmission and the vesicles are presumed to be the preterminal 'units' or 'packages' of transmitter substance predicted from the results of Fatt and Katz (1952). An attempt to explain the origin of vesicles has been made (Van Breemen et al., 1958); after ligation of frog sciatic nerves axonal swellings appeared, proximal to the ligatures, consistent with theories of axoplasm flow; vesicles accumulated in these swellings. This was accepted as evidence that the

vesicles move down the axons from the perikarya and it has been suggested that they originate in the Golgi complex. An alternative suggestion has been put forward (Palay, 1958) that vesicles originate by budding from elongated tubular profiles of the same diameter which are occasionally seen in endings. These appear to be continuations of the endoplasmic reticulum, which is seen in both dendrites and axons.

In electronmicrographs the presynaptic membrane appears as a smooth, gently undulating, adielectronic line, approximately $55\text{\AA}$ thick. In one or more places on each terminal it exhibits increased density and irregularly increased thickness; usually the synaptic vesicles within the terminals are aggregated against the patches of denser plasmalemma. These focal synaptic complexes may represent the actual sites of impulse transmission across the junction. The subsynaptic membranes at these foci also appear to be differentiated from the general membrane (de Robertis et al., 1963). The membrane is thickened and more dense, as is its presynaptic counterpart, and overlies a web of filaments, or canaliculi, of about $80\text{\AA}$ diameter which extends into the postsynaptic cytoplasm for a varying distance; this has been called the 'sub-synaptic web'. It is also of interest to note that de Robertis and his co-workers have identified what they call 'intersynaptic filaments' connecting pre- and post-synaptic membranes in synapses of the rat cortex; this was presupposed by Held in 1905 (see Eccles, 1964) who was a proponent of the 'reticular theory'. There is no other evidence for the existence of these filaments.

Neuromuscular Junction

As was implied in the prior reference to work on the frog sciatic nerve, there are basic similarities between the prejunctional

components of the neuronal synapse system and the neuromuscular junction: all terminal fibres are devoid of the myelin sheath which is characteristic of many of the axons leading up to them and all endings contain vesicles. However, the dissimilarities are important and I would like to point them out here and in the next section: many of the conclusions or predictions made about synaptic activity are derived by extrapolation from results on the neuromuscular junction and these differences in structure and electrical properties are indicative that any direct extrapolation from one system to the other could result in some quite erroneous conclusions.

Motor endings do not have boutons terminaux, but have thin, non-myelinated terminal fibres which occupy "gutters", or hollows, of varying depth in a flattened-out heap of sarcoplasm and mitochondria, the motor end-plate. There are no 'synaptic thickenings' of the boundary membranes, but fine lamellae have been observed radiating into the sarcoplasm from the sarcoplasmic membrane lining the 'synaptic gutters'. Electron microscopy (see Couteaux, 1958) has revealed that these fine ribbon-like structures are infoldings of the sarcoplasmic membrane and that the axoplasm surface membrane remains completely external. These folds may run along the gutters or be transversely oriented, depending on species. At the point where the two membranes are apposed and form a synaptic 'compound membrane', it is difficult to determine exactly what belongs to each of the two membranes. However, examination of the junctional fold in the end-plate of the chameleon lizard (Robertson, see Couteaux, 1958) has offered the following picture. The synaptic membrane complex, about 500 to 700 Å thick, resulting from apposition, comprises five layers. The two very dense inner membranes which limit axoplasm and sarcoplasm, respectively, are separated from each

other by three other layers: a dense middle layer and two light layers. One of these light layers obviously corresponds to the light layer of the sarcoplasm surface membrane and the dense middle layer results probably from a fusion of the two dense outer layers of the axoplasm and sarcoplasm membranes.

Histochemical studies (Couteaux, 1958) have shown that the high cholinesterase activity of the motor end-plate is mainly located within the subneural sarcoplasm at the level of the postsynaptic membrane itself.

Part II

Electrical Phenomena at Junctions

The role of the chemical transmitter at the neuronal synapse and neuromuscular junction is to carry information from the prejunctional member to the post-junctional, which will 'interpret' it in one of the two ways which may be available to it: by an excitatory or an inhibitory response, expressed in electrical form.

Biological membranes, those of nerve and muscle being prime examples, exhibit differential permeabilities to the ions which are present in the intra- and extracellular environments and it is generally accepted that these ions are maintained in asymmetric concentrations about the membrane: this has been shown to be true for sodium and potassium ions by a number of workers and there have been suggestions that in certain tissues a chloride pump may be in operation. As a result of these asymmetries of concentration of and inequality of permeabilities to

these ions, a net electro-motive force is expressed through the membrane impedance as a 'membrane potential', which is expressed by the modified Goldman equation (Goldman, 1943; Hodgkin and Katz, 1949).

$$V = \frac{RT}{F} \ln \frac{P_K [K^+]_O + P_{Na} [Na^+]_O + P_{Cl} [Cl^-]_i}{P_K [K^+]_i + P_{Na} [Na^+]_i + P_{Cl} [Cl^-]_O} +$$

where V is the potential difference across the membrane (see Appendix I). P is the permeability of the membrane to the subscripted ion and the other terms are standard physico-chemical notation.

'Electrogenic' membranes are of two basic types: electrically inexcitable, non-regenerative and electrically excitable, regenerative. A characteristic of the latter type, which includes striated muscle membranes and axon membranes, is the property of rectification (see Appendix II). The end-plate of frog striated muscle does not exhibit regenerative activity (Takeuchi and Takeuchi, 1959); the neuronal synapse cannot be studied. It has been shown that the dendritic membrane of motoneurons is capable of regenerative activity (Araki and Terzuolo, 1962) and we must assume therefore, that the only true "receptor membrane" on the neurone is that typified as electronically dense, and thickened, in the subsynaptic part of the cell. These areas are so small that they cannot be effectively studied by electrophysiological methods, but by extrapolation from the observations on end-plates, it is assumed that the subsynaptic receptor is not electrically excitable.

In the resting state, in most tissues, the permeability to potassium ions is relatively much greater than to ions of a larger radius (in the hydrated state) particularly sodium and so the membrane appears as a "potassium electrode" - the membrane potential approximates to the potassium equilibrium potential, usually about 70mV, inside negative

with respect to outside (the potassium potential is usually about 15mV greater, but sodium in particular contributes, even in the resting state).

An excitatory response, in electrically excitable tissue, is typified by a transient rise in sodium conductance (determined by the permeability of the membrane to sodium, see Appendix I) and a consequent reduction of the membrane polarity, which, in certain cases overshoots zero and approaches the sodium equilibrium potential (see especially Hodgkin and Huxley, 1952). The adequate stimulus for this regenerative response is an induced depolarization to a certain threshold level, and in the physiological state, as applied to neurones and muscle fibres, is caused by electrotonic conduction of the electrical response of the receptor tissue at the synapse or end-plate.

At these receptor areas the excitatory transmitter substance induces a certain conductance change, by some means, such that the synaptic region is passively depolarized to a new equilibrium potential, the new value of the conductance and the potential being characteristic of the transmitter action. If a sufficient area of the synaptic membrane is affected, a large enough net inward current flows, complemented by an outward current in extrasynaptic areas, to depolarize electrically excitable tissue to the threshold for regenerative activity (see fig. 1).

Takeuchi (1963a) studied conductance changes at the frog's sartorius end-plate in response to nerve stimulation and to the application of acetylcholine by micro-electrophoresis and found the equilibrium potential and conductance of the region to be identical under both influences, adding electrophysiological evidence to the pharmacological that acetylcholine is the neuromuscular transmitter. Of course, this same observation has been made by other workers at neuromuscular junctions of other species. The nature of this conductance change is unknown in the

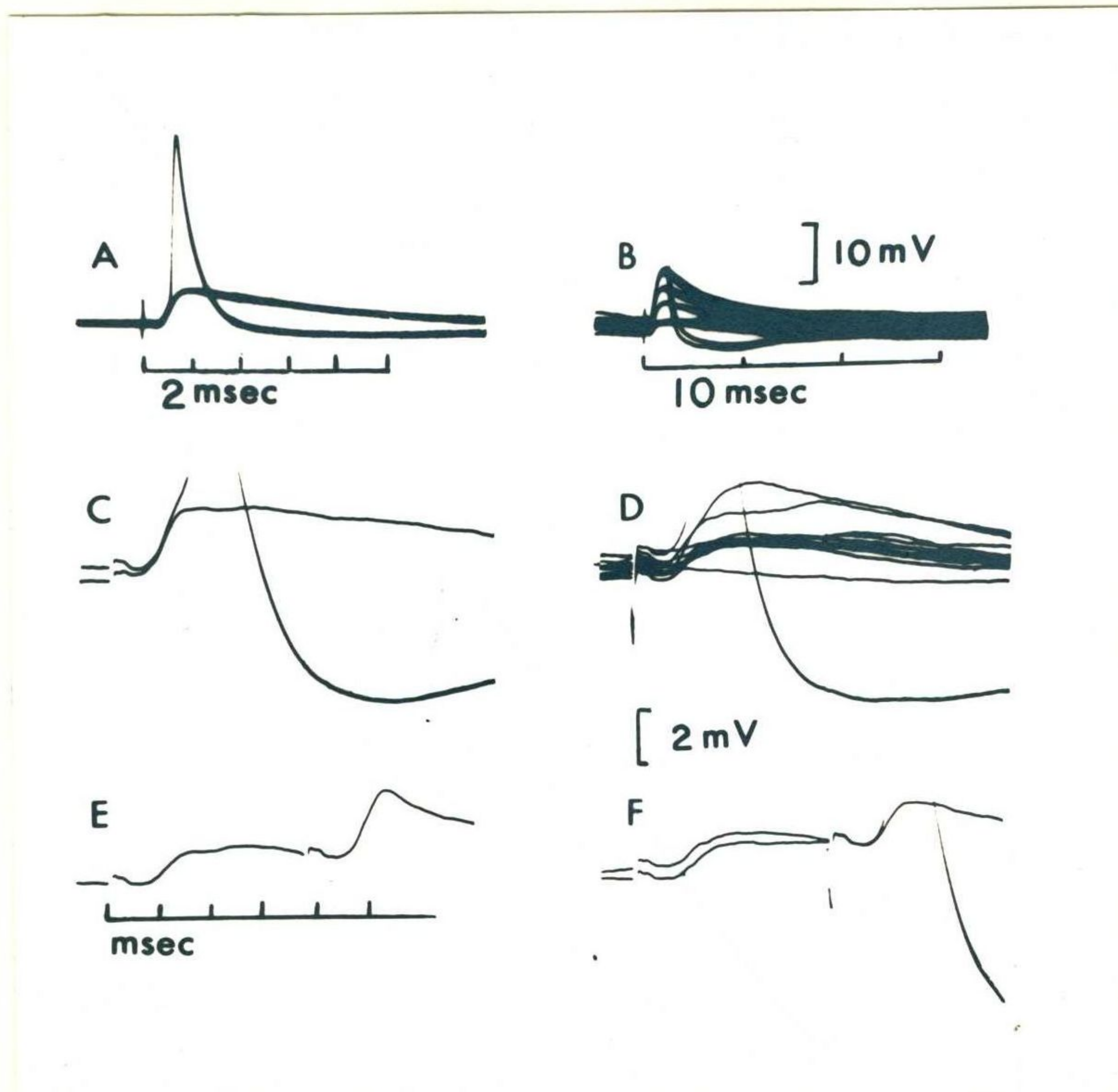


Fig. 1: Intracellular recordings from a red nucleus neurone. Davis and Tille. Unpublished observation. A. After the stimulus artifact (baseline 'blip') an e.p.s.p. is seen (low amplitude response) which is at threshold level and gives rise to occasional action potentials (large response). B. Recording during a period of stimulus gradation. The e.p.s.ps are of varying sizes. Suprathreshold inputs have resulted in spike discharges in the RN neurone, the falling and 'after-hyperpolarization' phases of which can be seen in the record. C. Record as in A using higher display gain. Note the 'after-hyperpolarization' phase of the spike. D. Record with input gradation. Note varying sizes of e.p.s.p. and single action potential. E. Two synaptic potentials elicited by closely spaced stimuli. The first has not fully decayed

at onset of second, which is potentiated, probably due to summation of transmitter concentrations (temporal summation). F. As E; an action potential has been triggered by the second response.

nerve cell, largely due to the difficulty of studying this system, as I have mentioned above, but certain observations have been made at the end-plate (Takeuchi, 1963 a, b) which may, or may not, be applicable to the neurone. A theory was held that excitatory end-plate currents may be due to a selective increase in sodium permeability, but this was incompatible with the results of del Castillo and Katz (1954 b) which were more reconcilable with a 'short-circuit' hypothesis, based on the concept that the end-plate region becomes 'sieve-like' under transmitter action. However, these authors admitted at that time, that there were insufficient data available to differentiate between a selective increase in permeability to several species of ions and a short-circuit-proper, attributable to a complete breakdown of a local ion barrier. Takeuchi's recent results have excluded any theories of chloride conductance changes during excitatory actions and have indicated that both sodium and potassium currents are involved, the relations between the 'reversal' potential and the external concentrations of sodium or potassium suggesting that the ratio of sodium and potassium conductances of the end-plate produced by acetylcholine is dependent upon the external potassium concentration. Continuation of the analysis indicated that calcium is involved, in that as external calcium concentration was increased the slope of the reversal (equilibrium) potential/external-sodium-concentration relation was reduced and the slope of the reversal potential/external-potassium-concentration was increased and that concomitantly there was a reduction

in the amplitude of the end-plate current and the total conductance change. Takeuchi concluded that increasing the external calcium concentration reduces the sodium conductance of the end-plate membrane produced by acetylcholine, with little or no effect on potassium conductance; this would appear to be true also of the electrically excitable segments of membrane, since an excitatory action on spinal cord neurons has been observed during the application of calcium chelators, iontophoretically (Curtis et al., 1960) which has been attributed to causing an increase in resting sodium permeability. Furthermore the end-plate membrane may become slightly permeable to calcium ion during acetylcholine action. Eccles (1964) cites results which tend to suggest that the end-plate conductance change might be subtended by a limited action in increasing the permeability of the subsynaptic membrane to cations, there probably being a size limitation with a diameter of hydrated ion no more than twice diameter of the hydrated potassium ion.

During the transmitter action the conductance of the end-plate may rise to 1 to 2×10^{-7} mhos, equivalent to a specific conductivity of 1 mho cm^{-2} which is many times the conductance of the muscle ('regenerative') membrane at the peak of the action potential. This dominance of the end-plate conductance tends to 'bleed' the action potential recorded in nearby regenerative tissue (fig. 2). This phenomenon, occurring at all chemically operated junctions is well illustrated in the figures of the paper of Blackman et al. (1963) depicting the effect of preganglionic nervous stimulation on the configuration of an antidromically fired action potential in the post-synaptic member, at various times after its initiation.

The alternative response to 'excitation', 'inhibition', is most important in the integrated function of the nervous system; the existence of inhibitory mechanisms affords a means whereby 'sophisticated' modulation of the influence of various inputs affecting some effector system may be attained. One of the earliest inhibitory mechanisms observed

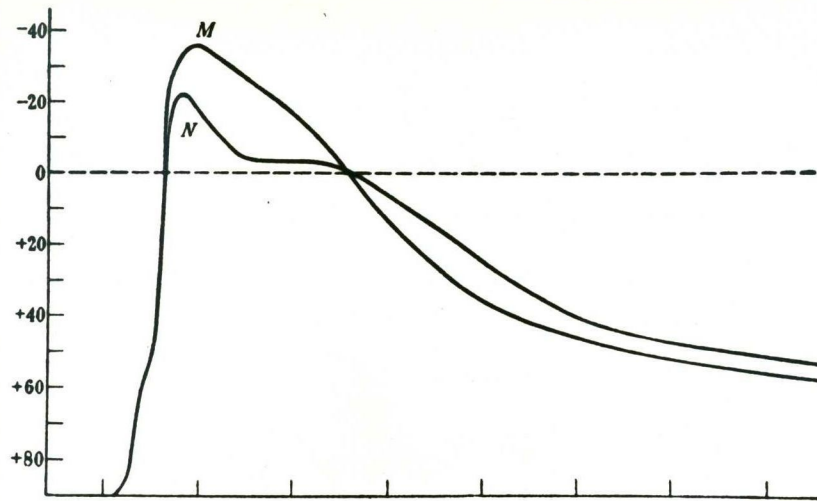


Fig. 2. From P. Fatt "The Biophysics of Junctional Transmission". Physiol. Rev., 34, 674-710 (1954)

Tracing 'M' indicates the time course of an action potential in muscle stimulated directly, 'N' that of an action potential recorded after nerve stimulation. As well as the peak-potential 'bleeding' referred to in the text, the passive return of potential to the resting level at the non-regenerative end-plate prolongs the duration of the spike falling phase.

was that of group Ia afferent fibres (from annulospiral endings on 'nuclear bag' intrafusal fibres) on motoneurones supplying antagonistic muscles (Lloyd, 1941). Subsequently a great deal of work has been done on central inhibitory mechanisms, particularly by the Canberra school of Eccles and he has reviewed the situation most thoroughly in several texts (see especially Eccles, 1964).

Inhibitory mechanisms have been accorded a great deal of interest and speculation for many years; since long before the recognition of inhibitory potentials. In 1925 (Howell, 1925) several mechanisms were propounded to explain inhibition in a number of types of tissue. Some of the premises accepted at that time have now been shown to be false or unlikely, for example "Inhibition is determined by the nature of the ending". We now recognize that one nerve cell may give rise to dichotomized axons, or axons with recurrent collaterals and according to Dale's principle both endings will secrete the same transmitter substance, yet it is conceivable that one ending may be excitatory to one cell and inhibitory to another. It is the nature of the receptor, rather than of the transmitter, which determines the type of response. This was, indeed, conceptualized by Howell, who in his discussion admitted "the reaction of the terminal mechanism may be chemical or physicochemical. The difference between an inhibitory termination and an excitatory termination may be due to some difference in the physical structure or properties of the junctional tissue, the synaptic membrane, or on the other hand there may be specific receptive substances each reacting to stimulation by the formation of a chemical product that in one case excites to functional activity and in the other brings about a state of inhibition ... it is possible that in any nerve cell, certain of the synapses may be excitatory and others inhibitory". We are accustomed to thinking in terms of an "inhibitory transmitter substance". Undoubtedly some substance may characteristically occur as the transmitter at inhibitory synapses, but the point to be made is that the inhibitory synaptic membrane responds, characteristically, to a substance which "fits its receptors": An "excitatory transmitter" would probably not elicit an excitatory response at these synaptic areas. An example of this "receptor specificity" has been demonstrated in the nervous system of the mollusc *Aplysia* (Tauc and Gerschenfeld, 1962). Two types of ganglion cell have been recognized in this preparation,

those having both excitatory and inhibitory synaptic input (designated H neurones) and those having only excitatory input (D neurones). Acetylcholine application caused hyperpolarization and inhibition of the former type and depolarization and excitation of the latter. Biochemical evidence that acetylcholine may be the "inhibitory transmitter" was supported by the finding that conductance and equilibrium potential changes induced by the ester were the same as those obtaining during the i.p.s.p. (inhibitory postsynaptic potential).

The postsynaptic inhibitory responses recognized to date in the mammalian nervous system have all been manifest in a hyperpolarization of the receptor and the areas under detectable influence according to the cable properties of the cell. Concomitant with this hyperpolarization is an increase in the conductance of the subsynaptic membrane as with an excitatory response, but there is no propagated inhibitory impulse.

The ionic mechanism of the inhibitory potential has been studied by numerous workers, most of them using cat motoneurones (e.g. Coombs, Eccles and Fatt, 1953, 1955), but extension of enquiry to other neurones which exhibit inhibitory postsynaptic potentials (i.p.s.ps) indicates that the underlying mechanism is probably common to all. It has been observed that the equilibrium potential of the i.p.s.p. has reversed, to a depolarizing response, during recordings in which chloride ions have diffused out of an intracellularly placed microelectrode filled with KCl, in cells of the dorsal spinocerebellar tract (Curtis, Eccles and Lundberg, 1958), hippocampal pyramidal cells (Andersen, Eccles and Løynning, 1963) and several others which have been studied. This, of course, as inspection of the equation given previously indicates, is interpretable as indicative of an increase of chloride permeability. However, in the original investigations (Coombs et al., 1953, 1955) electrophoretic injection of certain other anions also resulted in an alteration of the equilibrium

potential for the i.p.s.p. These were small anions, and large anions such as sulphate, phosphate, acetate and bicarbonate had no such effect. All ions causing changes in the i.p.s.p. did so with equal effectiveness - there was no "grading" of the effect; it is reasonable to assume that the effect of the transmitter on inhibitory receptors is to open a "channel" of precisely standardized size. This is supported by more recent experiments (Araki, Ito and Oscarsson, 1961) in which the anion permeability of the inhibitor-activated synapses of biceps and semitendinosus motoneurons induced by activation of quadriceps Ia afferents, and of certain unidentified motoneurons activated by polysynaptic pathways or by Renshaw inhibition evoked by supramaximal stimuli in L7 ventral root was studied. The same results were obtained for each of these differently induced i.p.s.ps. The results of these particular experiments indicated that the permeabilities to these anions is not increased during e.p.s.ps or action potentials: any changes observed in these phenomena were explainable by changes in the resting potential.

Normally, chloride is the only anion present in sufficient concentration to contribute to the inhibitory current. Measurements in the squid axon (Keynes, 1962) indicate that the chloride distribution is such that the equilibrium potential for chloride is about 39mV, inside negative, which is less than the resting potential. In other words the internal concentration is too high to be in electrochemical equilibrium and by the use of radiometric estimation of Cl^{36} fluxes with and without metabolic inhibitor (dinitrophenol) Keynes has established that there is an inwardly directed chloride 'pump'. If these data are applicable to mammalian neurones, the hyperpolarizing i.p.s.p. cannot be attributed solely to an increase in anion permeability. Recent work (Eccles, Eccles and Ito, 1964, reported in summary by Eccles, 1964) supports the theory that an

increased potassium conductance (E_K is probably 20mV or more, greater than the resting potential in the motoneurone) may play a role in i.p.s.p. generation.

In this discussion of electrical phenomena at junctions the concept of chemical transmission has been assumed and indeed the results are compatible with and supportive of such a concept. Results obtained from the ciliary ganglion of the chick (Martin and Pilar, 1963 a, b) are useful in pointing out the salient differences between electrical and chemical transmission, since this organ exhibits both phenomena.

An unusual morphological feature found in this ganglion is a caliciform termination of the presynaptic fibre on the ganglion cell. These components are sufficiently large to allow the introduction of microelectrodes and recordings have been obtained from pre- and postsynaptic sites during ortho- and antidromically initiated activity. During the recording of activity in some of the ganglion cells, following orthodromic activation, hyperpolarizing currents of increasing amplitude were passed into the cell until the action potential failed. During the phase of increasing the current, a hump appeared after the action potential, which eventually failed, leaving the hump, which was identified as an e.p.s.p., as described below. In some of the cells this 'e.p.s.p.' was preceded by a more rapid depolarization, which at resting potential gave rise to the spike. As the hyperpolarization was progressively increased, initiation of the spike was first transferred to the later "hump" and subsequently failed. This primary depolarization preceded the "hump" by 1.8 msec, consistent with expected synaptic delay at room temperature. With increasing hyperpolarization the "hump" became larger, appearing to have an equilibrium potential, whereas the primary depolarization was unchanged and obviously had no equilibrium potential. These observations were consistent with the interpretation that the primary depolarization was due

to electrical coupling with the presynaptic terminals, that the "hump" was an e.p.s.p., and that the cell membrane within the calyx is non-rectifying, at least with hyperpolarizing currents. A minute quantity of D-tubocurarine added to the bath blocked the e.p.s.p. but not the coupling potential and did not prevent the initiation of an action potential by the latter. Antidromic activation of the post-ganglionic (ciliary nerve) fibres resulted in trans-synaptic invasion of the preganglionic fibre (oculomotor nerve) indicating a bi-directional coupling: the action potential in the preterminal fibre following antidromic stimulation was frequently observed to elicit an excitatory response in the ganglion cell due to transmitter release.

Eccles (1961) has stated that efficient electrical coupling requires a relatively high cleft resistance in order to prevent the coupling current from escaping through the cleft, whereas chemical transmission requires a low cleft resistance so that the flow of current through the subsynaptic membrane is not impeded. The authors (Martin and Pilar, 1963a) pointed out that while the width of the synaptic cleft may be greater than would be expected for electrically coupled synapses (Robertson, 1955), the area of the ganglion cell covered by the calyx is large, providing a reasonable cleft resistance in spite of cleft width. Even with a large calyx the resistance of the membranes on either side of the junction may be several hundred times the effective cleft resistance, but the capacitative impedance seen by the coupling current may approach within an order of magnitude of the cleft resistance, depending on the rate of rise of the presynaptic spike, so that conditions favourable for electrical and chemical transmission may not necessarily be incompatible.

One more junctional phenomenon remains to be described: pre-synaptic inhibition. The history of this effect has origins in about 1933, in which year Gasser and Graham found that dorsal root volleys produced

slow positive potentials (P waves) of the cord dorsum and that the time courses of these waves corresponded approximately to that of the inhibition of flexor reflexes when one dorsal root volley was employed to condition the flexor reflex evoked by another volley. Recently (since 1961) the Canberra school has extensively studied central nervous mechanisms involving presynaptic inhibition (see Eccles, 1964) and it is interesting to see that, as Eccles points out, all examples studied have been effected on the synapses of the primary afferent fibres with the exception of the presynaptic inhibition of the secondary afferent fibres from the cuneate nucleus that relay in the thalamus (Andersen et al., 1963).

Presynaptic inhibition, as its title indicates, is not due to any action on the postsynaptic membrane which invariably occurs, as manifest in conductance changes, in postsynaptic inhibition. Recognition of the mechanism of the phenomenon is dependent upon data discussed in the previous section, that the amount of transmitter released by a nerve impulse is dependent upon the "availability" of transmitter and upon the height of the action potential invading the presynaptic terminals. In presynaptically inhibited junctions the e.p.s.p. is markedly reduced, presumably due to a reduction in transmitter release which is brought about by depolarization of the preterminals by the transmitter of other terminals which synapse onto them. Gray (1962) has presented electronmicrographs (see also Eccles, 1964, fig. 5A) of presumed presynaptic inhibitory synapses found in the upper lumbar region of the spinal cord of the cat. The salient features of the micrographs are (i) an apparently somatic or dendritic profile being synapsed onto at three sites by (ii) a structure with typical terminal inclusions - mitochondria and vesicles - in turn synapsed onto by (iii) two smaller structures of similar morphology. Eccles (1964) does not think it likely that presynaptic inhibition is significant "at stages of the afferent pathway after the primary afferent relay,

and a thorough examination of the cerebral cortex has disclosed no sign of synaptic structures ... that would subserve presynaptic inhibition".

CHAPTER II

PHARMACOLOGICAL STUDIES ON BRAIN MECHANISMS

At the present time, pharmacological studies have not been successful in characterizing transmitters at brain synapses, due almost entirely to the complexity of the system and to the difficulty of performing any definitive studies on it. The synapse of the axon collaterals of alpha motoneurons onto Renshaw cells in the spinal cord is the only junction in the mammalian central nervous system for which it can be claimed with any certainty that the transmitter substance has been elucidated (Eccles, 1962).

The development of the method of direct extracellular applications of drugs has increased the probability of more transmitters being discovered, although the task is still not an easy one. Prior to the introduction of electrophoretic administration from micropipettes (Nastuk, 1953) drugs were usually administered intravenously, intra-arterially or intrathecally. In some cases topical application or interstitial injection of potentially active material was also attempted. These techniques may be of use in psychopharmacology, but afford no means of studying the effects of drugs on single neurones or discrete neurone systems. As Curtis (1962) points out, 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 the observations. Firstly, there may be physical and chemical barriers restricting the access of the administered compound to an active site on the neurone. The former may be exemplified by the so-called "blood-brain" and "blood-cerebrospinal fluid" barriers. These "barriers" are presumably constituted by structures of varying chemical nature in which drugs will have greater or lesser diffusion coefficients and in consequence will enter the neuronal environment more or less readily when compared with one another. The "blood-brain barrier" is virtually absolute for some substances (Cf. Curtis and Eccles, 1958b)

and is remarkably "discriminating", the amine precursors dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (5-HTP) entering the cerebral tissue rapidly from the bloodstream, whilst the products of their metabolism, dopamine, noradrenaline and 5-hydroxytryptamine are impermeant. Rightly or wrongly, and certainly having no real evidence, Curtis and Eccles (1958b) assumed that "it is highly probable that the blood-brain barrier is associated with the capillaries and their covering sheaths". Enzymic systems may metabolize a compound before it reaches a proposed site of action destroying its activity or converting to another compound with a specific biological activity of its own. There is a further difficulty: the exact time of arrival at reactive receptors cannot be directly determined since the interval from time of administration to time of response is quite long in comparison to the time required for the transmission of information within the nervous system. As a result it is difficult to establish that the observed effect was caused by action of the drug on the neural structure under immediate investigation, rather than indirectly through action of the drug on peripheral structures or at other central sites, or even as an epiphenomenon of concomitant cardiovascular, respiratory or metabolic alterations.

Attempting to circumvent these difficulties Eccles (1947) tried to investigate the status of acetylcholine as a transmitter in the spinal cord of the frog by abolishing cholinesterase activity with prostigmine and then soaking the isolated oxygenated cord in strong acetylcholine solutions, his rationale being that if acetylcholine is a synaptic transmitter setting up a synaptic potential and hence a discharge of impulses from nerve cells, then it would be predicted that this transmission is depressed when a steady background concentration of acetylcholine

exists at the synapses and that the synaptic potential is virtually abolished by further increase in this concentration. However, he was unable to demonstrate such depression and concluded that acetylcholine does not mediate synaptic transmission in the frog's spinal cord. More recently results have been obtained indicating the probable presence of cholinergic transmission in the frog cord (Mitchell and Phillis, 1962).

Recently more refined techniques, especially that of micro-electrophoresis, have been developed to facilitate the testing of substances upon single nerve cells by applying them directly into the extracellular environment. This technique relies on the use of near-saturated solutions of drugs, in an ionized form, of sufficiently high conductance to pass current without boiling. In adjusting the pH of solutions to obtain optimal conductivity a lower limit of 3, or even higher, is adhered to due to the excitatory action of hydrogen ions themselves on neurones (Curtis, Phillis and Watkins, 1961).

Certain criteria have been tabulated which must be satisfied before any confidence can be placed in results which would classify a substance as a synaptic transmitter (Paton, 1958). The substance must exist in sufficient quantities in the presynaptic terminals which must also contain a synthesizing enzyme system; stimulation of the presynaptic nerves must release the substance in adequate quantities from the presynaptic terminals; the action of the substance on the postsynaptic cell must be identical with that of the synaptic action, particularly when applied by microelectrophoretic techniques; when the action of drugs is tested by microelectrophoretic injection the pharmacology of the synaptic transmission and of the postsynaptic action of the substance must be similar. It is this latter criterion that may be most easily satisfied: in fact the evidence that Renshaw cells are synaptically activated by acetylcholine is entirely pharmacological (Eccles, Eccles and Fatt, 1956). It should be noted here that there is further evidence

(Curtis, Phillis and Watkins, 1961b) pointing to the existence of an additional noncholinergic transmitter which is released upon these cells following excitation of dorsal root afferent fibres.

Pharmacological studies on Renshaw cells (Curtis and Eccles, 1958 a, b) indicate that the acetylcholine receptors of these cells are predominantly nicotinic in character. This could be interpreted in such a way as to extend the scope of Dale's principle. It is reasonable to assume that nerve cells of any particular type act at all of their synapses by liberating the same chemical transmitter ("Dale's Principle", Eccles, 1962). This principle may plausibly be related to the metabolic unity of a single cell, which is believed to extend even to the most remote branches. Alpha motoneurones exert their excitant influence on their terminal effector-organ (skeletal muscles) by means of a cholinergic mechanism (the neuromuscular junction): this same mechanism obtains at the junction of the axon collateral from these cells and the Renshaw cell. If the pharmacological nature of these two junctions is 'nicotinic', this could also be significant. After denervation of the frog sartorius muscle, the area sensitive to acetylcholine increases and in completely denervated fibres, ten weeks after nerve section, involves the whole fibre surface (Miledi, 1960). Acetylcholine applied for five days to denervated, isolated muscles does not abolish their supersensitivity. Functionally, the denervated end-plate is no different from the innervated tissue, with respect to miniature potential parameters and the author concluded that the motor nerve exerts a direct restraining influence on the chemo-sensitivity of the muscle. Such an influence is probably not mediated by acetylcholine. After denervation it is not the muscular inactivity, but the removal of this neural influence which causes super-sensitivity. If this influence were also responsible for determining the chemical conformation of 'receptors', one would expect to find nicotinic receptors on the Renshaw cell.

Acetylcholine will diffuse away from a structure as small as an end-bulb so rapidly that its concentration will be negligible in one second after its liberation. However, on the surface of Renshaw cells, when the cholinesterase has been inactivated, the acetylcholine transmitter action declines at a rate that is thousands of times slower than the calculated time considering the free diffusion rate, a single volley in the motoneurone axon collaterals giving rise to a repetitive high frequency discharge of a single Renshaw cell which may persist for at least 50 milliseconds (Eccles et al., 1956). This observation has been interpreted as indicating that there is some barrier in or around the synaptic cleft which prevents the free diffusion of the acetylcholine and this in turn has been used to explain the slowness in onset of effect of drugs affecting cholinergic mechanisms when applied electrophoretically (Curtis and Eccles, 1958 a, b). This concept has not been substantiated in any electron-microscope examinations: all synaptic clefts open directly into the extracellular spaces. However, there is no reason to expect that the shape of the cleft remains constant (Palay, 1958); during discharge the terminal may alter in size and shape and may even approach the post-synaptic membrane more closely: this would explain the prolongation of transmitter action (Eccles et al., 1956) if the discharged transmitter were effectively sealed off in the synaptic cleft. This could not explain the results of Curtis and Eccles (1958 a, b) unless such changes in cleft morphology were caused by transmitter substance, or its analogues, applied from without, as well as within, the synapse.

In the decerebrate, unanaesthetized cat spontaneously active units have been observed in the caudate nucleus which respond to iontophoretic applications of acetylcholine with increased rates of discharge (Bloom et al., 1964). It is significant that the caudate nucleus exhibits intense staining for specific acetylcholine-esterase activity (Koelle, 1954),

a high synthetic potential for acetylcholine (Feldberg and Vogt, 1948) and that the basal ganglia as a whole exhibit a very high concentration of tissue acetylcholine (MacIntosh, 1941). Supporting this evidence and offering what would appear to be final proof of the existence of cholinergic synapses in the caudate is the isolation of acetylcholine-containing "synaptosomes" or pinched-off nerve ending particles (NEPs) separated from homogenates of the nuclei from dogs (Lavery et al., 1963) by differential centrifugation. The great difficulty here, as in other centres of the brainstem and its associated structures, is to determine which tracts passing by way of the anatomically and functionally complicated nuclei contain the synaptic transmission system which has been virtually proven to exist.

The cerebral cortex is well provided with the enzymes required for the synthesis and destruction of acetylcholine (Hebb, 1957) and it can be shown that large quantities, which are apparently related to the functional state of the brain as indicated by the electrocorticogram, can be liberated in it continuously if precautions are taken to inactivate cerebral cholinesterases (Mitchell, 1963). Iontophoretic application of acetylcholine (Krnjević and Phillis, 1963a) to the cerebral cortex of cats, rabbits and monkeys excited an average of 15% of the cells to which these applications were made. The majority of these cells were found in or near the primary sensory areas, at a depth of 0.8-1.3 mm and characteristically occurred in groups. Many of these cells can be excited by direct afferent activity from the thalamus, by transcallosal volleys, or by stimulation of medial thalamic nuclei, but do not appear to be specifically associated with any of these pathways: this again is an indication of the difficulty of anatomical and functional location of the synapse. Betz cells in the pericruciate region appear very sensitive to acetylcholine and in certain respects, such as their distribution

in depth and their behaviour, they are typical of cortical cholinceptive cells in general. Further pharmacological studies (Krnjević and Phillis, 1963 b) made it evident that cortical acetylcholine receptors have a strongly muscarinic character. Cholinceptive cells are not markedly different from other cortical neurones with respect to the excitatory and inhibitory actions of various amino acids and catechol and indole amines.

Studies in other areas, particularly the thalamus, have yielded results suggestive of the presence of cholinergic excitatory synapses, but here again the evidence is inconclusive and if such synapses are present, the pathways in which they may be involved cannot yet be decided. Curtis and Davis (1963) applied a series of choline esters to cells of the lateral geniculate nucleus, identified by synaptic responses to volleys in the low- or high-threshold contralateral optic nerve fibres or to light-evoked impulses from the ipsilateral eye. These agents, with the exception of carbachol, failed to activate quiescent cells, but facilitated 'background' discharge and synaptic activity. The features of this excitant activity made it unlikely that there is a similarity between these compounds and the excitatory transmitter which is released by optic nerve terminals. Firstly, synaptic activation was suppressed by 4- and 5-HT, but the excitatory effect of acetylcholine was unaffected by the indolealkylamines. If the site of action of these tryptamine derivatives is postsynaptic, preventing the access of excitatory transmitter to specific receptor sites (Cf. Curtis and Davis, 1962), this observation contradicts either an interaction between quaternary ammonium compounds and these specific sites, or the release of transmitter as a consequence of a direct action of the excitants upon the presynaptic terminals. Of course, if the action of 4- and 5-HT were purely presynaptic, blocking the release of transmitter, acetylcholine could well be the transmitter, its action at the postsynaptic receptor after iontophoretic application

being unimpeded. However, dihydro-B-erythroidine failed to block the action of acetylcholine; this evidence would itself preclude acetylcholine's being an optic-geniculate transmitter as it constitutes a failure to meet one of the definitive criteria. It should also be noted that there is very little choline acetylase in the lateral geniculate region (Hebb, 1957) and although choline esterases are present (Burgen and Chipman, 1951) the comparatively prolonged effect of acetylcholine on geniculate neurones indicates the relative lack of importance of these enzymes in its removal after application.

The ventrobasal (Andersen and Curtis, 1964 a, b) and ventrolateral (Davis, Rowe, Kelly, 1964) thalamic nuclei have both been found to contain cells excitable by acetylcholine. The acetylcholine receptors in ventrobasal thalamus have been described as intermediate in type between the muscarinic cortical receptors and the nicotinic Renshaw cell receptors and are similar to those in lateral geniculate and in the medullary reticular formation (Salmoiraghi and Steiner, 1963). Andersen and Curtis found that acetylcholine fired most ventrobasal relay neurones (identifiable by firing antidromically from the primary sensory cortex) whereas the depolarization of thalamic interneurones remained sub-threshold. Unable to identify the actual pathway which may have a cholinergically mediated synaptic mechanism, Andersen and Curtis pointed out that the action of acetylcholine upon a particular neurone will depend upon the number of appropriately sensitive synapses and revised the conclusion drawn on lateral geniculate results (Curtis and Davis, 1963). It was, at that time, suggested that the acetylcholine sensitivity may have been associated with the presence of non-optic synapses and this was discounted due to the apparent inability of the fibres within the nucleus to meet the neurochemical criteria necessary

to be satisfied, by convention, before a substance can be considered to play a role as synaptic transmitter: the pharmacological differences between geniculate and Renshaw cells also prompted this conclusion. It is now thought (Andersen and Curtis, 1964b) that this conclusion was unwarranted and that the relatively weak action of acetylcholine on geniculate cells is explainable by assuming that cholinergically mediated synapses may be present in sparse population: it is even suggested that such synapses may have a common origin to those upon ventrobasal neurones. A characteristic of both ventrobasal and ventrolateral thalamic activity is "spindle" discharges: this spindle activity is unaffected by acetylcholine and compounds affecting cholinergic mechanisms. Davis et al. (1964) found the postsynaptic activity in ventrolateral thalamus evoked by brachium conjunctivum volleys to be unaffected by the cholinergic blocking compound dihydro-B-erhythroidine, although this drug blocked the increase in 'spontaneous' background discharge evoked by application of acetylcholine.

Cells responsive to the application of acetylcholine with an increase in their firing rate have been found in the hypothalamic area, mainly in the paraventricular and ventral-median nuclei (Bloom et al., 1963). Neurochemical evidence (Hebb and Silver, 1956) is supportive of the possibility of the amine playing a role in synapses here, although Bloom et al. are mindful of the amine's having a non-synaptic chemical function.

Excitatory responses to acetylcholine have been reported in other areas, in which the likelihood of the amine having a normal function at the synapse is very small. A study of the superior and inferior colliculi and mesencephalic and medullary reticular formations (Curtis and Koizumi, 1961) yielded sensitive cells only in the inferior colliculus, most of these cells responding to auditory stimuli. From

Hebb's (1957) results cholinergically mediated synaptic activity is not expected in the colliculi: apart from responding to sound, no further identification of cells was made. The action of acetylcholine on collicular cells was not blocked by dihydro-B-erythroidine and it is unfortunate that a comparative test was not made on a synaptic mechanism: cells to which DHBE was applied failed to respond to orthodromic stimulation, when no drug was present. Since at most other known, or assumed, cholinergic junctions this compound blocks the action of applied acetylcholine to some extent, it appears unlikely that the iontophoretically applied ester was interacting with cholinergic synaptic receptors upon the neurones: however, the question of whether or not acetylcholine is a transmitter in this region must be left open.

An inhibitory response elicited by acetylcholine has also been observed in hypothalamic neurones (Bloom et al., 1963) olfactory bulb cells of three histological types (tufted, mitral and granule) (von Baumgarten et al., 1963) and medullary neurones (Salmoiraghi and Steiner, 1963; Bradley and Wolstencroft, 1962). This duality of response may be related to basic differences in the chemical receptor membranes (see previous chapter) or to mediation of effects through a neighbouring interneurone, or through axon terminals. The inability to record intracellularly during the application of drugs to these cells has not enabled an analysis of membrane phenomena to be carried out and consequently a concept that acetylcholine may normally operate as an inhibitory transmitter in the central nervous system cannot be substantiated.

Inhibition:

The nature of the chemical transmitter substances for both pre- and postsynaptic inhibition remains unknown, although attempts have been made to isolate transmitters from central nervous tissue.

As indicated previously, postsynaptic inhibition in the mammalian central nervous system, as it so far has been recognized has been marked by a hyperpolarization of the receptor cell with apparent increases in the potassium and chloride conductances at the subsynaptic membrane. Presynaptic inhibition is a phenomenon apparently due to a slow, sustained depolarization of the terminal fibres which results in a failure of the boutons to release excitatory transmitter, due to reduction in the overshoot of the invading action potential.

The requirements to be met before a substance can be classified as an inhibitory transmitter have been tabulated (Curtis, 1961) and are essentially the same as those set up for the recognition of an excitatory transmitter.

Factor I, which has been extracted from mammalian brain (Florey and McLennan, 1955 a; Elliott and Florey, 1956) mainly the grey matter of the "extrapyramidal system" (Florey and Florey, 1958), depresses spinal monosynaptic reflexes when applied topically to the cord (Florey and McLennan, 1955 b; Honour and McLennan, 1960). This depression is transient and may be followed by potentiation: polysynaptic reflexes are increased in magnitude. It has been reported that topical application of factor I to the spinal cord hyperpolarizes motoneurons, reduces the magnitude of i.p.s.ps and e.p.s.ps and blocks orthodromic spike production (McLennan, 1961) and in consequence it is not impossible that factor I is an inhibitory transmitter acting upon spinal motoneurons.

γ -amino-n-butyric acid (GABA) is sometimes present in extracts of factor I (Cf. McLennan, 1961). This amino acid has been shown to have a potent blocking action on the crayfish stretch receptor neurone and to depress spike potentials of motoneurons, dorsal horn cells and Renshaw cells, the postsynaptic potentials both excitatory and inhibitory and to decrease membrane electrical excitability without

altering resting potentials (Curtis et al., 1959). This and other evidence indicates that GABA has a "non-specific" depressant action upon the whole surface membrane of neurones, both the chemically activated subsynaptic regions and the remaining electrically excited postsynaptic membrane. Curtis (1963) considers it therefore unlikely that GABA (or the related compound γ -amino-B-hydroxy-butyric acid, GABOB) are transmitters for postsynaptic inhibition within the mammalian spinal cord, although he does not discount the possibility that it might be functional in presynaptic inhibition.

Pharmacological studies on neurones, in which indole and catechol amines have been applied by the electrophoretic technique have revealed a depressant effect which may or may not be indicative of a role in synaptic or presynaptic inhibition.

Neurochemical studies, indicating that the DOPA-5HTP decarboxylase activity is extremely low in the lateral geniculate nucleus and other parts of the visual pathway (Kuntzman et al., 1961) except the superior colliculus make it unlikely that catechol or indole amines subserve any synaptic function in the lateral geniculate, in spite of the observation that intracarotid and intravenous injection of lysergic acid diethylamide reduced postsynaptic responses of neurones in the lateral geniculate nucleus of the cat, produced by optic nerve impulses (Bishop, Burke and Hayhow, 1959). The compound failed to affect the spike potentials of presynaptic fibres, but it increased synaptic delay. Repetitive stimulation of the optic nerve overcame the block, indicating a probable interference with the attachment of the natural excitatory transmitter to its appropriate subsynaptic receptors. The known antagonism of lysergic acid diethylamide (LSD) towards the action of 5-hydroxytryptamine on smooth muscle (Gaddum, 1958) was suggestive that this amine, or some closely related compound, could be the transmitter.

application of 5-hydroxytryptamine to the extracellular environment of geniculate neurones (Curtis and Davis, 1962) blocked the transmission of optic nerve impulses to the geniculate cells, although it did not affect the antidromic activation of the latter, and was ineffective in depressing the activity evoked by the application of glutamate ions which, like other mono-amino dicarboxylic acids, depolarize and cause repetitive discharges of neurones (Cf. Curtis, Phillis and Watkins, 1960). Catecholamine and related phenylethylamine derivatives were also found to block orthodromic activation of geniculate neurones: the authors did not report any findings on the amino acid-evoked discharge. It is interesting to note that the geniculate synapse was the first central neuronal junction for which a specific blocking agent was found; the tryptamine derivatives and noradrenaline failed to block the activation of spinal neurones (Curtis and Davis, 1962) and brainstem cells (Curtis and Koizumi, 1961).

The caudate nucleus is a rich source of the endogenous amines acetylcholine (see previous section), dopamine (Bertler and Rosengren, 1959) and possibly 5-hydroxytryptamine (Kuntzman et al., 1961). Iontophoretic application of noradrenaline and dopamine to spontaneously active caudate neurones resulted in depression of the discharge rate with noradrenaline and an equal increase and decrease with dopamine (Bloom et al., 1964). While the apparent duality of action of dopamine and not of noradrenaline may be due to a sampling bias, the result for dopamine itself is interesting. In view of its implication in basal ganglia disease (see General Introduction) it is difficult to assume that its presence in the caudate nucleus in high concentration is atavistic, especially since this nucleus is a phylogenetically recent structure and a dual mode of action would appear unlikely, unless the cells which were depressed and those which were excited were of a different histological and functional type. Thus the question of the function of catecholamines

in caudate, whether they have some synaptic function as inhibitory transmitters, or as excitants, or whether they (especially dopamine) fulfil some other, non-synaptic role, remains open.

The electrophoretic administration of noradrenaline and 5-hydroxytryptamine to hypothalamic neurones (Bloom et al., 1963) resulted in a decrease in spontaneous discharge of cells diffusely distributed throughout the hypothalamus: the results are of course compatible with a possible functional role for these endogenous amines in this structure which is particularly rich in both compounds.

In spite of these numerous studies, possibly the only sites where a role for catechol- or indole- amines as synaptic inhibitors have possibly been located are in the olfactory bulb of the rabbit and in the spinal cord of the cat. After the original studies on olfactory bulb (von Baumgarten et al., 1963) in which endogenous amines were found to depress the spontaneous firing of all three cell types present (tufted, mitral and granule), further investigation (Bloom et al., 1964; Salmoiraghi et al., 1964) has indicated that noradrenaline may be the transmitter responsible for inhibition of mitral cells by stimulation of the lateral olfactory tract (lateral olfactory tract inhibitory response, LIR). By means of iontophoretic administration of amine antagonists and drugs that deplete monoamine stores, a close functional relationship between noradrenaline and LIR was made: dibenamine iontophoresis sufficient to block neuronal inhibition by noradrenaline also caused significant shortening of LIR. Blockade of the acetylcholine effect by its antagonists was achieved, but they had no effect on LIR, and LSD and BOL, antagonists of 5-HT were more effective in blocking the noradrenaline effect than that of the indole compound. Drug-induced noradrenaline depletion also caused significant shortening of LIR. Intravenous injection of dihydroxyphenylalanine (DOPA) or 5-hydroxytryptophan (5-HTP) into acute spinal cats inhibited transmission from the flexor reflex afferents (FRA) to primary

afferents, motoneurones and ascending pathways (Andén, Lundberg, Rosengren and Vyklický, 1963). Further studies (Andén, Jukes and Lundberg, 1964) indicated that DOPA probably acts through the formation and liberation of noradrenaline, not of dopamine, since the action of DOPA was blocked by the prior administration of reserpine. Tests with monoamine oxidase and decarboxylase inhibitors on the effect of 5-HTP indicated that its action on motoneuronal excitability is caused entirely by monoamine (5-HT) liberation. It was postulated that the action of DOPA and at least part of the action of 5-HTP depends on monoaminergic nerve terminals into which these precursor substances enter and cause increased synthesis and overflow of monoamines. A more detailed electrophysiological investigation (Andén, Jukes, Lundberg and Vyklický, 1964) led to the postulation of the existence of an inhibitory mechanism, probably mediated by noradrenaline, suppressing short latency flexor reflex afferents actions and 'releasing' delayed flexor reflex afferents effects. The reason for the appearance of the delayed flexor reflex afferents actions was probably the depressed transmission in the short latency paths from the flexor reflex afferents, the inhibition releasing neuronal paths that are normally concealed in the spinal state by inhibition from the short latency reflex paths. The authors pointed out that there may be similar reflexes that are normally concealed in the acute spinal preparation and where release depends on a particular descending control system.

Catecholamine and Indole-amine Distribution and Localization in CNS

Although the evidence in support of the concept is not unequivocal, there is a strong general contention that certain biogenic amines may subserve a role as neurohumoral transmitters in synapses of the central nervous system; as has been pointed out in the General Introduction, dopamine has been biochemically implicated in the function of the basal ganglia.

In 1954 Vogt carried out the first major investigation on the distribution of central noradrenaline, using the brains of dogs and cats killed by rapid exsanguination following chloroform anaesthesia, finding highest concentrations in the hypothalamus and the lowest in areas such as the visual and acoustic cortices (5% the hypothalamic content) and the posterior roots, with only 1%.

Subsequent to the discovery that the noradrenaline precursor, dopamine is also present in high concentrations in certain areas of the central nervous system several re-assessments of amine distribution have been made. After some preliminary reports Bertler and Rosengren (1959) published their findings from several species. In all the mammalian brains investigated noradrenaline and dopamine were present in about the same amounts. Noradrenaline was found localized mainly to the brainstem while, as stated in the General Introduction, dopamine was concentrated in the corpus striatum. A particularly thorough investigation of the human brain (Bertler, 1961) revealed concentrations very similar to those in other animals, the values for noradrenaline and dopamine in the red nucleus being almost equal (0.22 and 0.19 ug/g) and very close to the values for whole mesencephalon in other species.

An investigation of decarboxylase activity and distribution of 5-hydroxytryptamine and noradrenaline in various parts of the cat brain (Kuntzman et al., 1961) resulted in the findings that DOPA-5HTP-decarboxylases (assumed by some workers to be the same enzyme) are

present in high activity in the brainstem, the reticular formation, the hypothalamus, the basal and intralaminar portions of the thalamus: in general, those areas which are mainly involved in co-ordination of behavioural, autonomic and somatic activity and that the "high-concentration areas" for 5-HT overlap those for dopamine and noradrenaline.

Vogt stated in 1957 that in spite of the evidence which she and others had accumulated regarding the distribution of brain "sympathin" the question of whether the amines are present in neurones or in other cells, for example the glia, remained unanswered. That the latter cellular system may form part of the "blood-brain barrier" is widely recognized and in parts of the brain where the arterioles are richly innervated with adrenergic vasomotor fibres we might expect to find some amines in the glia, but recent evidence shows clearly that the amines themselves contain the greater part of, if not all, the catecholamines (Carlsson et al., 1962).

That brain catecholamines may be taken up from the circulation is largely precluded by the findings reported by Weil-Malherbe (1960) that the hypothalamus is the only part of the brain which takes up a significant amount of noradrenaline, even two hours after infusion into the general circulation.

Teams of investigators in Sweden have been very active in studying all aspects of catecholamine metabolism and localization in the adrenal medulla and "adrenergic" neurones. In 1956 von Euler and Hillarp concluded, as a result of their observations that adrenergic axons, which contain "catecholamine granules" as do adrenal medullary cells, are in certain respects analogous to the latter. This opinion has been confirmed following many investigations and the realizations of this duplicity has made biochemical studies on the mechanisms of drug action, etcetera, simpler than if there were two discrete systems to

be analysed. A fluorescence histochemical method has been developed (Falck, 1962; Falck et al., 1962) with which catecholamine and indole-amine localization at the cellular level can be achieved. The method involves the removal of the brain from an animal immediately after death and the excision and freezing in liquid-nitrogen cooled propane of the areas selected for study. The specimens are freeze-dried at -35°C for 8-10 days and treated with formaldehyde at $+80^{\circ}\text{C}$ for one hour. During this treatment the catecholamines and 5-hydroxytryptamine are prevented from diffusing through the tissue and condense with the formaldehyde, forming intensely fluorescent complexes which are not extracted by hot paraffin or xylene. Preparations are then infiltrated with paraffin in vacuo at $+60^{\circ}$ for ten minutes and sections are mounted in Entellan (Merck). The fluorescence is preserved quite well for up to two days in this medium. After fluorescence examination, the fluorescent material in the sections can be localized by staining with gallocyanin-chromalum or toluidin blue.

Carlsson et al. (1962) observed varicose fibres containing catecholamines, which exhibit green to yellow-green fluorescence with the method described above, in four circumscribed and bilaterally symmetrical areas within the hypothalamus:

1. A large area just below (and sometimes extending slightly above) the lateral part of the anterior commissures
2. The supraoptic nuclei
3. The paraventricular nuclei
4. An area in the walls of the third ventricle (periventricular nuclei) within the anterior hypothalamus

These findings, incidentally, are in keeping with Carlsson's et al. earlier (1960) observation that the majority of the hypothalamus' noradrenaline is in the anterior two thirds.

Fluorescent products were localized in fine fibres running between and enclosing the nerve cells which themselves - like the glia cells - show no fluorescence. These fibres were very fine and did not show up well in photomicrographs; they were widely scattered in the hypothalamus but were scarce in the posterior region and were entirely absent in the suprachiasmatic nuclei and in the optic nerves, chiasma and tracts. They were quite dense in the preoptic region and in a large area just posterior to the paraventricular nuclei. Carlsson et al. (1962) had earlier made the observation that these fibres have the same characteristic appearance as the adrenergic nerve terminals in peripheral tissues with small, intensely fluorescent enlargements dispersed at irregular intervals. The fluorescent material appeared to be localized within the fibre and was not present down its full length, which was only rarely more than 0.1 mm. The thickness of the varicosities was usually 0.3-0.8 μ and seldom more than 1 μ ; the fibres themselves were only seen, in many cases, because of the fluorescence, although some thicker ones were found in the anterior hypothalamus. The fibres were not ordinary adrenergic nerves to blood vessels, since bilateral cervical sympathectomy did not cause them to disappear; furthermore, no adrenergic nerve supply to vessels within the hypothalamus of the mouse or rat has been observed, whereas such nerves are constantly and easily detectable in the vessels at the base of the brain.

The fibres seemed to run to and partly enclose nerve cells on which the termination appeared to be with a small knob; in nuclei with dense fibre accumulations the fibres appeared to form real "baskets" around the nerve cells. From observations of the less dense areas it appeared that a fibre approaches a cell and then becomes directly superimposed and terminates on the perikaryon. It was immediately obvious that the fluorescent fibres represent terminal parts of axons forming synaptic contact with nerve cells. In all respects hypothalamic and peripheral 'adrenergic' terminals showed the same characteristics.

Median Eminence:

Fluorescence here was not confined to discrete fibres but existed as a dense accumulation, forming a continuous and distinct zone, which was localized to the superficial part of the nervous tissue where the primary plexus of the hypophyseal portal system arises. It began in the rostral part of the median eminence and continued down into the hypophyseal stalk. The outer borderline was indented by the portal vessels and coincided with the external surface of the nervous tissue. The fluorescence faded diffusely towards the interior parts of the brain; irregular, faintly fluorescent stripes radiated towards the infundibular recess of the third ventricle, in the lateral walls of which, above the median eminence (arcuate nuclei) is a group of small nerve cells which exhibited a fairly weak green fluorescence. Big nerve cells forming a large but scattered group in the posterior hypothalamus and pons exhibited a strong green to yellow-green fluorescence. Green-yellow to yellow fluorescence, indicative of tryptamines was observed in very fine smooth fibres located in several circumscribed areas, such as regions surrounding the pre-optic area anteriorly, laterally and dorsally, and in the medial part of the amygdala.

Fuxe (1963) using the technique of Falck, has obtained similar results from a study of the cat and guinea pig.

The fluorescent varicose fibres in the hypothalamus have the same characteristic appearance and must - as judged from the fluorescence intensity - accumulate and store similar very high concentrations of noradrenaline to the 'terminals' in peripheral tissues. Besides the morphologic evidence there is biochemical evidence for the view that the fluorescent fibres represent the terminal parts of the axons. The very high accumulation of noradrenaline in these terminal parts is just what would be expected if they were terminals belonging to adrenergic neurones in the brain.

Caudate Nucleus:

No distinct fluorescent fibres or nerve cells have been found in the caudate nucleus of the rat and the mouse, but the nucleus as a whole developed a marked yellow-green to green fluorescence which stood out well against the dark brain regions surrounding it; the fluorescence was diffuse and somewhat uneven. Small, irregular areas with quite high intensity were present everywhere whilst in contrast to this the nerve cell bodies and fibre bundles fluoresced very faintly or not at all.

Monoamines have also been localized in fibres in the spinal cord of the mouse and rat (Carlsson et al., 1964) using the histochemical method of Falck. The highest accumulation of fluorescent fibres was present in the sympathetic intermedio-lateral column, most, if not all, of the nerve cells here being surrounded by terminals. The fibres have the same characteristic appearance as the noradrenergic nerve terminals in peripheral tissues and the synaptic terminals containing noradrenaline in the hypothalamus. In the mouse terminals containing 5-hydroxytryptamine seemed to occur most frequently, whereas there was a preponderance of noradrenaline-containing fibres in the rat. It is not known, however, whether the two kinds of fibres terminated on the same nerve cells or on different ones.

In the ventral horn many fibres with both green (noradrenaline) and yellow (5-hydroxytryptamine) fluorescence were present, being most abundant in the ventral and ventro-lateral part, especially in the cervical and lumbar enlargements. Some of them seemed to make intimate contacts with and to terminate on dendrites and cell bodies of large motoneurons. Some fibres of both kinds appeared also to terminate on nerve cells in the ventral part of the posterior (dorsal) horn; the vessels in the spinal cord, like those within the brain, did not usually contain adrenergic nerves.

Studies on rat brain homogenates (de Robertis et al., 1962) in which the subcellular localization of acetylcholine and acetylcholinesterase was examined, revealed that non-cholinergic endings, differentiable from cholinergic by density gradient centrifugation as well as by histochemical examination, had about double the protein-content of the acetylcholine-containing fraction. This was in agreement with the general concept that there are more non-cholinergic endings in the brain than cholinergic.

At about the same time as de Robertis and his colleagues' work was being done, an attempt was being made to differentiate 5-HT-containing fractions from acetylcholine-fractions of guinea pig brain homogenates (Michaelson and Whittaker, 1962). Although the peak activities of Ach and 5-HT were found in two different density layers after differential centrifugation, there was a complete overlap, the fractionation not resulting in a sharp separation of particles into two chemically or morphologically distinct types. Endings were present which apparently contained neither Ach nor 5-HT. Shortly afterwards Ryall (1962), using the centrifugation technique of de Robertis et al. (1962), obtained results suggesting that the particles which contain 5-HT may be different from those which contain Ach. Michaelson and Whittaker (1963) reaffirmed these earlier findings, that 5-HT is principally found in a particulate fraction free of myelin and mitochondria and identifiable as consisting largely of pinched-off nerve endings. The forces binding 5-HT to its "storage sites" were found to be quite labile relative to those binding acetylcholine: the monoamine oxidase inhibitor iso-propyl-nicotinic acid hydrazide did not alter the subcellular distribution of 5-HT, even though the level that could be extracted was increased. The results at this stage, while not proving that 5-HT is a transmitter, were consistent with such a function and strongly suggested a localization of bound amine in the nerve endings. The alternative possibility existed that particulate 5-HT is located in

storage organoids distinct from nerve endings, but similar in sedimentation and density characteristics, even in complex gradients, such a possibility being difficult to disprove. Michaelson et al. (1963) carried out some experiments which clarified the picture a little more, confirming that 5-HT and noradrenaline are differently located to Ach, but could not distinguish between, nor define the sites of localization of these two amines. The coup de grace has been delivered by de Lorenzo's group (Maynert et al., 1964) who isolated a vesicular fraction containing noradrenaline and 5-hydroxytryptamine from disrupted nerve-ending particles from rat brain - stem. During their experiments they investigated a wide range of conditions of hypotonicity and sonification to disrupt the NEPs. Under optimal conditions the vesicular fractions obtained by ultrasonic or hypotonic disruption contained about 25% of the noradrenaline, 20% of the 5-HT and 10% of the protein in the crude NEP preparation. Electron-microscopic examination revealed that the vesicular fractions from both ultrasonic and hypotonic disruption were composed almost entirely of vesicles of about the same size and appearance as those seen at synaptic junctions. In fixed and sectioned material the dense-core vesicles often associated with adrenergic nerve endings (for references see Maynert et al., 1964) were exceedingly rare. At least three morphologically distinct kinds of vesicle could be observed in negatively stained preparations. They were differentiated primarily on the basis of the thickness of their walls, although one type was distinguishable by a matrix containing many small granules. Vesicles extruding a granular material were observed rather frequently. The vesicles of this study were different from the noradrenaline-containing particles prepared from splenic nerves by von Euler (1958), in terms of their sedimentation characteristics and by the same criterion, from 5-HT granules of the dog's duodenal mucosa, which sedimented with mitochondria (Baker, 1958) and from those of blood platelets (Buckingham and Maynert, 1964). Electronmicroscopic examination of the heavy ('debris') sediment failed to reveal granules of the

expected appearance, but because of the morphological complexity of this fraction the authors admitted that their presence could not be unequivocally denied. As noted above, one of the vesicle types (designated 'type 3' in their paper) appeared to contain several small granules: moreover, vesicles apparently extruding a granular material were observed rather frequently in negatively stained preparations and Maynert et al. (1964) admit that "if these are valid interpretations of the micrographs, a new dimension is added to the problem of the tissue storage of chemicals like acetylcholine, noradrenaline and 5-HT. It is possible that the tiny granules rather than the vesicles themselves may be the ultimate bound forms of certain chemical mediators".

On the basis of this admission it is not impossible that the brain granules are chemically and physically similar to the large granules isolated from peripheral adrenergic nerve fibres and adrenal medulla. Those still within their vesicles would not sediment with the heavier fractions because of the vesicles' characteristics and those which had been liberated by disruption, leaving vesicle 'ghosts' could well have been indistinguishable among the heavier debris pellet contents, as suggested by the authors.

It is interesting that de Robertis and Vaz Ferreyra (1957, see de Robertis, 1961) have observed that when adrenal medulla cells are stimulated through the splanchnic nerve the granules pass towards the cell membrane and one finds them in greater numbers in the region of the membrane. The granule membrane (von Euler and Hillarp, 1956) then seems to coalesce tangentially with the cell membrane which appears to rupture at the point of contact so that the granule contents are discharged.

THE RED NUCLEUS

CHAPTER III

The Anatomo-Physiological Context

The red nucleus is an ovoid mass of grey matter which, largely due to its considerable vascularity, appears pinkish in fresh brain. In transverse sections this nucleus presents a circular outline and can be followed from the level of the inferior border of the superior colliculus to the hypothalamus. Afferent fibres project to the red nucleus chiefly from the deep cerebellar nuclei and from the cortex of the frontal lobe of the cerebral hemispheres. These fibres, most abundant on the medial surface of the nucleus, tend to encapsulate it. Postsynaptic ('rubral') fibres project deep in the spinal cord, to brainstem nuclei, the cerebellum and the diencephalon (see Fig. 3).

The deep cerebellar nuclei are usually considered as three units: the fastigial nucleus (closest to the midline), the interposed nucleus (a collective of the globose and emboliform nuclei) and the dentate nucleus (lying most lateral to the midline). Studies in several species (see especially Carpenter and Stevens, 1957; Carpenter, 1959; Cohen, Chambers and Sprague, 1958; Carpenter, Brittin and Pines, 1958; Foltze and Matzke, 1960; Jansen and Jansen, 1956; McMasters and Russell, 1958) indicate close interspecies similarities with regard to sources, projections and relations of cerebello-efferent fibres.

Functional cerebellar cortico-nuclear relations are indicated by clearly defined patterns of projection of Purkinje cells from the cortex onto the subjacent deep nuclei (Eager, 1963). The major tract in which cerebello-efferent fibres project from the cerebellum to its brainstem relations is the brachium conjunctivum, a fibre bundle composed of the axons of cells in the deep cerebellar nuclei. Contrary to the findings of Carpenter, Brittin and Pines (1958), Cohen, Chambers and Sprague (1958), using the Nauta technique to study retrograde changes following lesions

of the deep cerebellar nuclei, found that all cerebellar nuclei contribute fibres to the brachium. Fastigial efferents also leave the cerebellum in a tract known as the uncinate fasciculus (Carpenter et al., 1958) and descend to medullar and pontine nuclei ipsi- and contralateral to the fastigial origins. The projections and connections of the fastigial nuclei are very complex, extending in the brainstem from the medulla to the rostral thalamus, and a great deal of confusion is evident in the literature. Consequently, fastigio-efferent fibres are not shown in figure 3.

Rinvik and Walberg (1963) have demonstrated a significant projection from cerebral cortical areas to the red nucleus, pointing out that it is somatotopically organized and discuss the likelihood that cerebral influences on motor performance may be transmitted by a cortico-rubro-spinal pathway as well as by direct cortico-spinal projections.

In an extensive series of degeneration experiments in the cat Hinman and Carpenter (1959) mapped the projections of the red nucleus in the brainstem and spinal cord and the reader is referred to their paper for detailed descriptions. They demonstrated descending fibres terminating at medullary levels, some crossed fibres (probably collaterals from the rubrospinal tract) terminating in the "supraspinal part" of the lateral reticular nucleus and some uncrossed fibres ("rubrobulbar tract") terminating in the "supraspinal part" constituted largely by the dorsal lamellae, of the inferior olivary complex. These findings confirmed those of earlier workers. The rubrospinal tract descends right to sacral segments and its fibres appear to terminate on cells in the anterior horn, down the whole length of the cord. No rubrofugal fibres have been reported terminating in motor nuclei of the cranial nerves.

The lateral reticular nucleus, according to Walberg (1958) receives fibres only from the red nucleus, although Cohen et al. (1958) found it to receive a fastigial projection. This nucleus sends fibres

to the flocculo-nodular lobe and the hemispheres of the cerebellum, these reticulo-cerebellar projections being ipsilateral to the red nucleus origins. The inferior olive, on the other hand, receives a mixed supraspinal input, thereby probably subserving some integrative function (Walberg, 1956, 1958) and sends fibres to crus I of the cerebellar hemispheres. Decussating fibres from the red nucleus also ascend the superior cerebellar peduncle in close apposition to the efferent brachial fibres and project to the deep cerebellar nuclei, particularly the dentate, in which the brachium fibres originate (Carpenter, 1957). Thus the red nucleus is equipped to feed information back to the cerebellum by three different paths, the fibres projecting to quite different terminations, a system obviously subtending the integrative function of the cerebellum.

The demonstration that rubrospinal tract fibres terminate on cells in the anterior horn of the cord, as well as its anatomical membership of the 'extrapyramidal' system, immediately implicates the red nucleus in the supraspinal control of motor function.

Carpenter (1961) points out that the subthalamic and red nuclei are most commonly associated with dyskinesia in man, but that in spite of the attribution of several varied motor disturbances to lesions of the latter nucleus it is probably only transient ataxia, tremor and asynergia and enduring hypokinetic phenomena which are subtended by rubral destruction and that these effects are probably due to dysfunction of the rubrospinal tract. These conclusions were preceded by experiments (Carpenter and Brittin, 1958) in the rhesus monkey in which discrete brainstem lesions were produced and the concomitant motor effects observed. During these experiments transient choreoid dyskinesias were seen in animals with red nucleus lesions and concomitant destruction of some brachial fibres which did not form a link in the cerebello-rubro-spinal pathway. It was concluded that these dyskinetic phenomena resulting from lesions of the deep cerebellar nuclei or brachium

conjunctivum are not dependent on the integrity of the red nucleus or rubrospinal tract.

As is shown in fig. 3, the red nucleus projects fibres through the subthalamic nucleus, some of which terminate (not shown) in this cell group. Lesions of this nucleus cause persistent choreoid activity. The subthalamic nucleus projects fibres to the globus pallidus which must be intact for the hyperkinesis to be observed and the results are interpreted (Carpenter, 1961) as being due to a release of the globus pallidus as a consequence of the subthalamic lesion. The discharge of the globus pallidus is mediated via pallidofugal fibre systems to the lateral reticular nuclear groups of the thalamus (connections not shown in fig. 3) which in turn probably alter neural activity at the cerebro-cortical level. Corticospinal tract fibres mostly decussate in the lower medulla, so that the physiological disturbances are expressed in the contralateral part of the body (Carpenter, 1961). The release phenomena observed with cerebellar lesions would appear to involve contralateral diencephalic nuclei receiving crossed brachial fibres. Disordered information projected to the cerebral cortex on that side would be relayed to segmental levels by the cortico-spinal tract which, as described above, decussates in the medulla, so that cerebellar lesions are manifest in ipsilateral dyskinesia. Lesions of the red nucleus or rubrospinal tract ameliorate the hyperkinesis, but the dyskinesias are still present (Carpenter and Brittin, 1958).

At the present stage observations of this type are useful in the interpretation of clinical signs but do not give any insight into the physiological mechanisms involved.

While still not at an 'advanced' stage of development, the understanding of the mechanisms of rubral control of peripheral motricity has improved markedly in the last few years, following improved physiological definition of the innervation of the muscle spindle and extrafusar

muscle fibres and advances in the understanding of the function of the spindle.

Appelberg (1962 a, b) showed that stimulation of the red nucleus depresses the activity of gamma motoneurons and that the static response of primary afferents is decreased whereas the 'dynamic' response is increased. This is interpreted as indicating individual supraspinal control of gamma₁ (to 'nuclear bag' intrafusal fibres, responsible for the 'dynamic' primary afferent response, see Appendix III) and gamma₂ (to 'nuclear chain' intrafusal fibres, responsible for 'static' primary response) motoneurons: this observation is equally important for an understanding of muscle spindle function as of the function of the red nucleus (see P.B.C. Matthews, 1964). Eldred (1955, as cited by Appelberg, 1962a) demonstrated gamma-motor effects caused by stimulation of the interposita and dentate nuclei, but Appelberg (1962a) considers the effect he (Eldred) observed to be transmitted through bulbo-reticular pathways, and the red nucleus to relay influences to the gamma system from cerebral centres. Subsequent investigations (Appelberg and Kosary, 1963), in which different areas of the red nucleus were stimulated, indicate that this nucleus influences fusimotor neurones differently in different areas, according to a somatotopic organization within the nucleus. Stimulation of the dorsal part caused inhibition of gammas to both hind-limb flexor and extensor muscles, with concomitant lowering of the afferent 'static' discharge, as described above. Stimulation of the ventral portion of the nucleus facilitated flexor spindles, but inhibited extensors; facilitation of alpha-motoneurons (motor to extrafusal fibres) in hind-limb flexor muscles and inhibition of those to extensors was also observed. That the rubrospinal tract conveys impulses to alpha-motoneurons from cerebellar origins has been strongly supported by the results of Thulin (1963) and of Manni et al. (1964), among others. Thulin showed that

repetitive stimulation of the red nucleus caused mixed facilitatory and inhibitory effects on both flexor and extensor motoneurons: Manni and his coworkers obtained both facilitatory and inhibitory hind-limb extensor alpha effects and varying forelimb postural changes from stimulation of the interpositus nucleus which projects to the red nucleus; these results are applicable to an analysis of the motor effects observed by Pompeiano (1959).

In review it can be said that we are aware of the red nucleus[†] being a link in central circuits controlling motor function and that some of the effects of central stimulation are explainable in terms of information transmission by two independent, but functionally correlated partners (the alpha and gamma motor systems), both of which are at least partially dependent on rubral integrity.

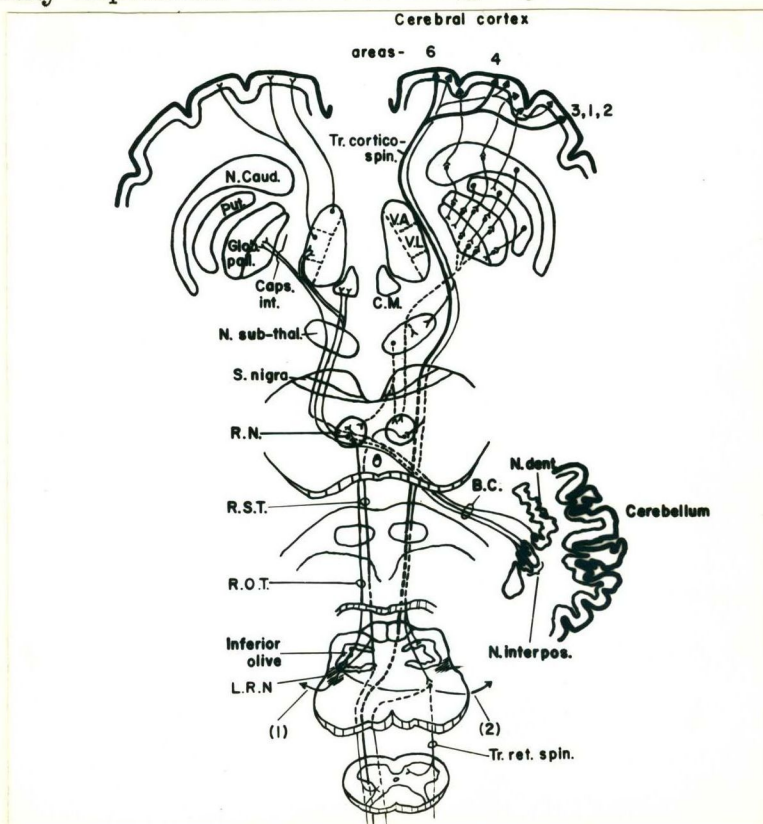


Fig.3. Connections of the red nucleus. Fibres ascend from (1) the lateral reticular nuc. and (2) the inferior olive to the cerebellum as described in the text.

Methods

i) Preparations:

Two series of experiments were carried out on cats.

The first, in which 47 cells were examined in 9 animals, used 8 animals anaesthetized with pentobarbital sodium (Sagatal, May and Baker) which was initially administered intramuscularly (30 mg/kg) and then intravenously as required and one preparation anaesthetized with chloralose (70 mg/kg), 9 cells being examined in this animal.

The second series used 3 unanaesthetized animals decerebrated by a precollicular trans-section of the brainstem: 18 cells were examined in these preparations.

ii) Surgery:

Surgery was performed on the animals to be used in the anaesthetized state after an initial intramuscular injection of barbiturate: those to be decerebrated were initially anaesthetized with diethyl ether (induction) and maintained on methoxyflurane (Penthrane, Abbott) during surgical procedure. Gaseous anaesthesia was discontinued at least two hours before recording procedures were begun.

A polyethylene cannula with Luer-lock syringe fitting was introduced to the brachial vein for intravenous injection procedures and a polyethylene endotracheal tube (T-type) was inserted to provide a short, unobstructed airway and to facilitate the maintenance of decerebrate animals with positive-pressure respiration.

Dorsal midline incisions allowed the attachment of clamps to the lateral faces of the iliac processes and to a mid-thoracic spinous process by which the animals were suspended in a close-to-normal posture in the 'cat-frame'. This method of holding the cats, in contrast to the method of supporting them in a V-trough, placed no unusual

pressures on the abdominal viscera and allowed normal thoracic respiratory excursions.

After midline incision extending from approximately the level of the third cervical vertebra, over the cranium, almost to the dorsal margins of the eye-orbits and retraction of the skin and muscle, parietal and occipital craniectomies were performed to provide entry for recording and stimulating electrodes, respectively. The open sinuses in the bone were packed with bone-wax and the dura mater was removed from both exposed surfaces. Usually the parietal craniectomy was bilateral (see fig. 4): the superior sagittal sinus was sealed with the diathermy instrument and removed and the cerebral tissue was removed by suction, often to the depth of the dorsal surface of the colliculi (see fig. 5) so as to reduce the amount of tissue through which glass electrodes had to be advanced before entering the red nucleus.

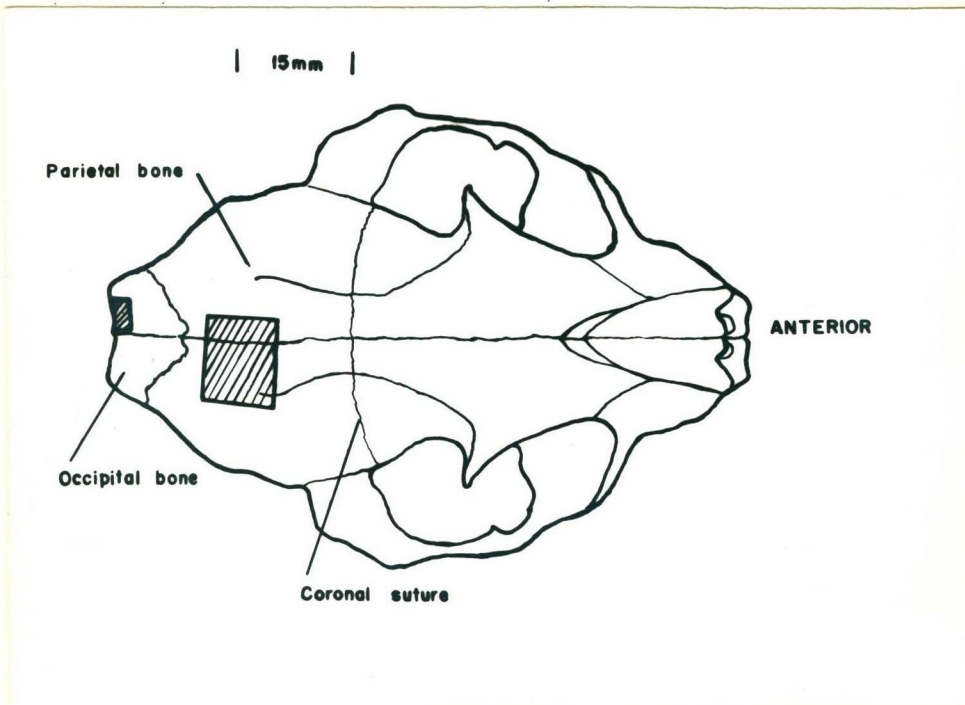


Fig. 4: Dorsal aspect of cat's skull. Cross-hatched areas mark the positions for craniectomy.

Decerebration was achieved by a trans-section of the brainstem just rostral to the colliculi and when this was successful the lightly anaesthetized animals exhibited the postural rigidity associated with the decerebrate state. The rigidity was suppressed with injections of gallamine triethiodide (Flaxedil, May and Baker) sufficient to keep the animals non-rigid and the animals were maintained with a positive-pressure respirator (see previous note).

The size of the cat's pupils was used as an index of the possible intactness of the red nucleus. Slit-like pupils were indicative of an intact Cr. N. III (oculomotor) nucleus, this nucleus lying dorsal and slightly posterior to the red nucleus, and therefore probably of the red nucleus itself.

The animal's temperature was monitored with a rectal thermometer and was maintained with a heater coil held against the underside of the body with a blanket pinned around the cat.

iii) Experimental technique:

The cat's head was fixed in a head-frame, an integral part of the 'cat frame' by means of moulded steel ear-plugs which were inserted deep into the aural canal. The head was spatially oriented as specified by the "Horsley-Clarke" stereotaxic co-ordinate system by lifting the front of the head, with the ear-plugs as axes, by means of a bar pressing against the roof of the cat's mouth, until it was firmly held against two horizontal bars, equidistant from the midline, making contact with the malar bones of the optic orbit.

The brachium conjunctivum fibres were subjected to bipolar electrical stimulation by means of a bank of five electrodes inserted through the 'hole' in the occipital bone with a micro-manipulator (see fig. 5). The electrodes consisted of straight steel sewing needles insulated, except for the tips, with a high-resistance lacquer (Monsanto Chemicals (Aust.) Ltd.) applied by dipping the needles and drying in an

oven at 90°C . The electrodes were arranged in two banks, of three and two, in such a way that each electrode was 1 mm from its nearest neighbour. Stimulating pulses were applied through a programmed switching unit and a stimulus isolation unit from a 90V dry cell source.

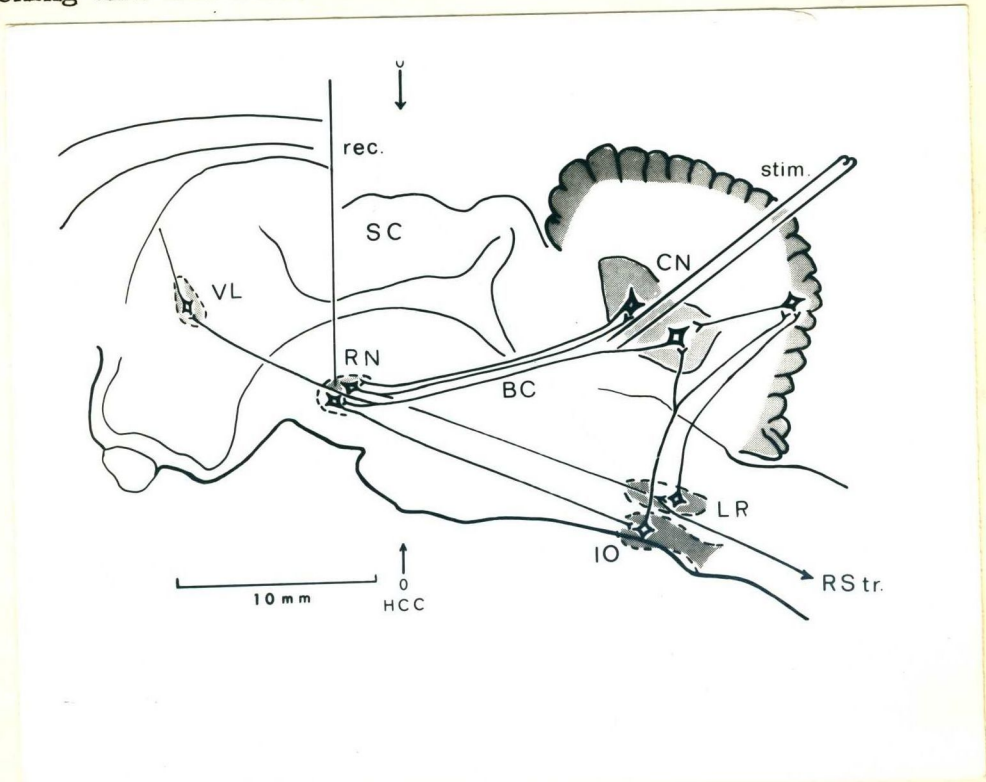


Fig. 5: Parasagittal section of caudal portion of cat brain showing brainstem and cerebellum. Stimulating electrodes (stim.) were advanced into the brachium conjunctivum (BC) arising in the cerebellar nuclei (CN). The cerebrum was removed to the depth of the superior colliculus (SC), allowing the tip of the recording electrode (rec.) to be nearer to the red nucleus (RN) before entering the tissue. RN is about 3.5 mm anterior to Horsley-Clarke zero (H.C.C.O). LR: Lateral reticular nucleus, adjacent to inferior olive, 10 mm posterior to H.C.C. zero. VL: Vento-lateral nucleus of the thalamus.

A suitable recording site in the red nucleus was found using a whittled monopole steel electrode (Grundfest et al., 1950; Bishop and Collins, 1950) insulated as were the stimulators, the electrode being inserted stereotaxically, using micromanipulators, according to the atlas of Snider and Niemer (1961). This steel electrode was replaced by 5-barrel glass micropipettes (overall diameter, 6-12 μ) for the pharmacological experiments.

Solutions of drugs in suitably ionized form were prepared in double-distilled water and filtered through cellulose filter discs (pore size 0.45 μ) (Millipore Filter Corporation) before being introduced to the electrode barrels. Most of the drugs used were available in the form of ionized salts and did not require adjustment, but the amino acids, in particular, were adjusted, the pH of their solutions being taken to 8 with sodium hydroxide, so that these compounds could be applied as anions. In no case was any solution made more acid than pH 3.5 since hydrogen ions themselves excite neurones (Curtis, Phillis and Watkins, 1961b).

The glass micropipettes were received as 'blanks' and when required were drawn to a fine tip in a programmed electrode 'puller' consisting essentially of (i) a small heating coil which softened the glass while the pipette was being drawn out by (ii) a solenoid-operated draw-bar. The drawn pipettes had an overall tip diameter less than 2 μ and before use this was increased to the above stated diameter by "knocking back" with a fine, flat-ended glass rod under a stereo microscope using a micromanipulator. One advantage of the multibarrelled pipette is that it affords a means of applying compounds to a region which is approximately geometrically the same as that from which recordings are being made, even though the two may differ in magnitude. However, to make the most of this feature it is essential that the

orifices are in the same plane: it was necessary, when knocking the tips back, to ensure that one barrel did not 'break back' further than the others as was prone to happen as the glass 'aged' after pulling, the fused joints especially becoming brittle. After a recording 'run' it was necessary to check the integrity of the tip: if one of the drug barrels broke during an experiment, records taken during the use of such a barrel would probably be erroneous.

Once the drug solutions and micropipettes were prepared, the solutions were introduced to the four outer barrels, 4M sodium chloride to the central recording barrel, and the tips filled by spinning the electrodes in a centrifuge at 3,500 r.p.m. (about 2200 x g at the tip) for seven to ten minutes (Curtis and Davis, 1962).

Drugs tend to escape from pipettes by two mechanisms:

(i) bulk flow, due to the pressure head of solution in the pipette barrel and (ii) diffusion. In order to try and prevent loss of drugs from the barrels a 'backing potential' of $1/2V$ was applied across the tips as soon as the electrode had entered the brain tissue. This 'backing potential' also helped prevent the efflux of a drug due to electrical coupling with a nearby barrel when this barrel was in use (see Curtis et al., 1959).

Recording Procedures

Rubral activity was monitored on a Tektronix Type 565 cathode ray oscilloscope following amplification of the signal by an a.c.-coupled differential preamplifier with cathode follower input stage. A d.c.-line was used to monitor the changes in tip, contact, etcetera potentials which had to be neutralized with 'bucking' units built into the recording circuit, since the a.c.-coupled amplifier 'overloaded' with a swing of 50mV or more: it was also necessary in monitoring intracellular recording (experiments not reported here).



A second oscilloscope, triggered by the first, was equipped with similar input stages and its ancillary equipment allowed voltage and time calibrating pulses to be displayed and photographed, along with the biological response, using a Grass Kymograph camera. The recorded signal was fed into a Hewlett-Packard 523 CR electronic counter, the trigger-level of which was manually set and continuously variable. Frequently more than one rubral unit was discharging and these could be discriminated by comparison of the sizes of the extracellular responses. It was possible, by setting the triggering level of the counter at a suitable value, to examine the behaviour of only the cells with the largest recorded response, the counter triggering a pulse generator, the output of which was displayed on the CRO. When the pulses displayed corresponded only to the responses to be counted, the trigger level was assumed satisfactory. The pulse generator also fed an integrating preamplifier which summed the voltage input in one second lots and displayed the integrals as an analogue of the firing rate on an Offner Dynograph Penwriter.

A block diagram of the recording system is shown in fig. 6.

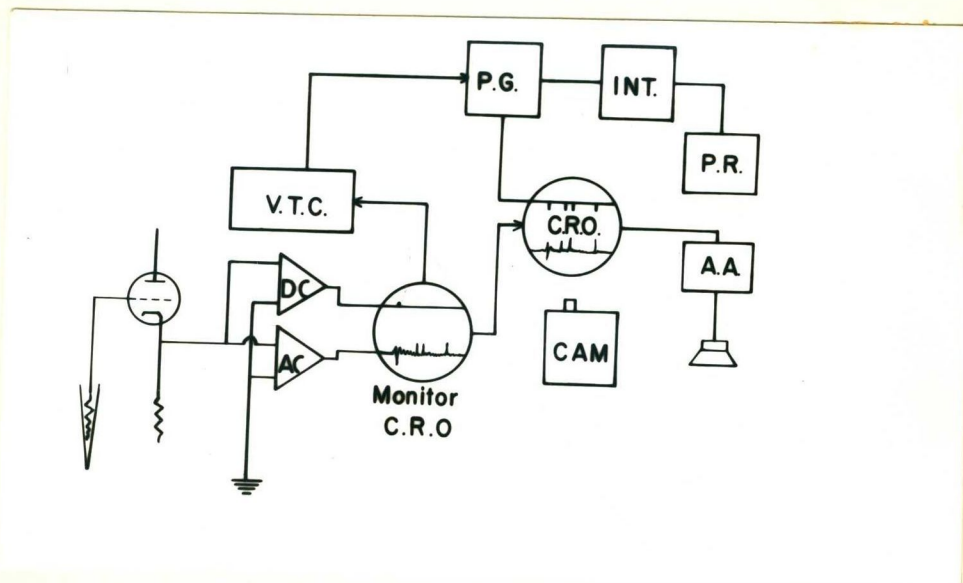


Fig. 6: Block diagram of recording system described in text. Lines with arrows carry trigger-pulses.

The ancillary equipment of the second oscilloscope, for calibrating pulses, is not shown.

C.R.O.: cathode ray oscilloscope

V.T.C.: Variable trigger-level counter (Hewlett-Packard)

P.G.: Pulse generator

INT.: Integrating preamplifier

P.R.: Amplifier-penwriter (Offner Dynograph)

CAM.: Grass Camera

A.A.: Audio-amplifier

Results:

i) Spontaneous Activity

Spontaneous neuronal discharges were recorded from fibres in the decussation of the brachium conjunctivum (Calma, 1952) and from rubral neurones (Massion and Albe-Fessard, 1963) in which it was demonstrated that "under light anaesthesia with nembutal (18 mg/kg) sensory stimulation does not produce any response in the magnocellular portion"¹ of the red nucleus, whereas sensory stimulation provoked an increase in the spontaneous activity already present in the chloralose anaesthetized cat. Rubral activity was shown to be very largely dependent upon the integrity of the cerebellum, and it has been assumed that the 'spontaneous' discharge of rubral neurones is mainly the result of activity in the brachium fibres.

In the present experiments, eighteen cells, all of which were synaptically evoked following a brachium conjunctivum volley, were studied in the decerebrate preparation. Fourteen cells exhibited 'spontaneous' firing at rates varying between 40 and 80 impulses per second; ten of them discharged regularly (fig. 8, Con.) while four displayed apparently random bursts of activity in which the discharge frequency reached 120/sec.

Although spontaneous firing was only seen in the decerebrate, unanaesthetized cat, some of the results obtained in the anaesthetized animals may reflect differences in the pharmacological properties of pentobarbitone and chloralose (see Discussion).

The use of two different preparations, the anaesthetized and the decerebrate animal, has afforded an opportunity of studying different aspects of rubral activity and has provided circumstantial evidence that

1. A free translation from the original French

phenylethylamines and 5-hydroxytryptamine have different actions on the synaptic mechanism.

Throughout this thesis the terms 'synaptic' and 'evoked' refer to rubral response to volleys, elicited by electrical stimulation, in the brachium and 'spontaneous' refers to rubral response to autogenic input, as seen in the unanaesthetized preparations.

2. Sensitivity of Rubral Cells to Amino Acids

In all experiments at least one of the four drug barrels was filled with a concentrated solution of either L-glutamate sodium or DL-homocysteate sodium so that adjacent neurones could be chemically evoked when required. At every site so far examined in the central nervous system it has been found that these two amino acids, as well as others of the monoamine dicarboxylic acid series (Curtis and Watkins, 1960) have excitant properties.

In the experiments reported here and in earlier investigations (e.g. Curtis and Davis, 1963) these compounds have assisted evaluation of the effect of substances which may alter a neurone's excitability, but which are too weak to either block synaptically evoked firing or to cause quiescent cells to discharge. The procedure involves the production of a constant neuronal firing frequency (e.g. 50/sec) by the regular applications (e.g. 15 sec. 'ON', 15 sec. 'OFF') of a steady amount of the excitant amino acid alone by passing a constant current (e.g. 30nA) through the electrode. Continual addition of the test substance during the 'ON-OFF' sequence of amino acid applications allows any change in membrane excitability to be observed, manifest in an increase or decrease of the chemically evoked discharge rate.

Tested rubral cells all fired repetitively in response to applications of either amino acid. Homocysteate applications were clearly the more potent, the current required to produce a similar firing rate

to that provoked by a certain current of glutamate being only a quarter to one third as much.

Figure 7A shows the results of a typical comparison of the two amino acids when applied separately to the one cell at various current strengths. It appeared that homocysteate excitation was slower in onset and offset than the glutamate effect, as has been remarked by Curtis for spinal interneurons (including Renshaw cells) and cells of the ventrobasal thalamic nucleus (see Andersen and Curtis, 1964a). A synergistic action was observed (fig. 7A, arrow) when the two were evoked at the same time.

Gamma aminobutyric acid (GABA) was shown to be a potent depressant of neuronal activity in the red nucleus as in every other site so far examined in the CNS. It suppressed firing of rubral cells in response to glutamate excitation (fig. 7B) and to synaptic activation from the brachium conjunctivum.

GABA has been found to have a depressant action on a wide variety of tissues. It has been found in extracts of human brain containing Factor I (Cf. McLennan, 1961; see Chapter 2, 'Inhibition') and was considered as the inhibitory transmitter acting on the crustacean abdominal stretch receptor neurone, although this was rendered unlikely by the findings of Florey and Biederman (1960). Experiments on the slowly adapting receptor cells of the crayfish indicated a membrane hyperpolarization in response to GABA application (Kuffler and Edwards, 1958), increasing with GABA concentration, until the membrane potential reached that of the normal inhibitory equilibrium potential and i.p.s.ps disappeared. At near 'threshold' for block, GABA not only reduced the i.p.s.p. but also accelerated its falling phase, just as it did the after positivities of antidromic impulses, indicating an action of increasing conductances to specific ions, it was claimed by the authors, in dendrites and cell body, since concentrations even 1000 x

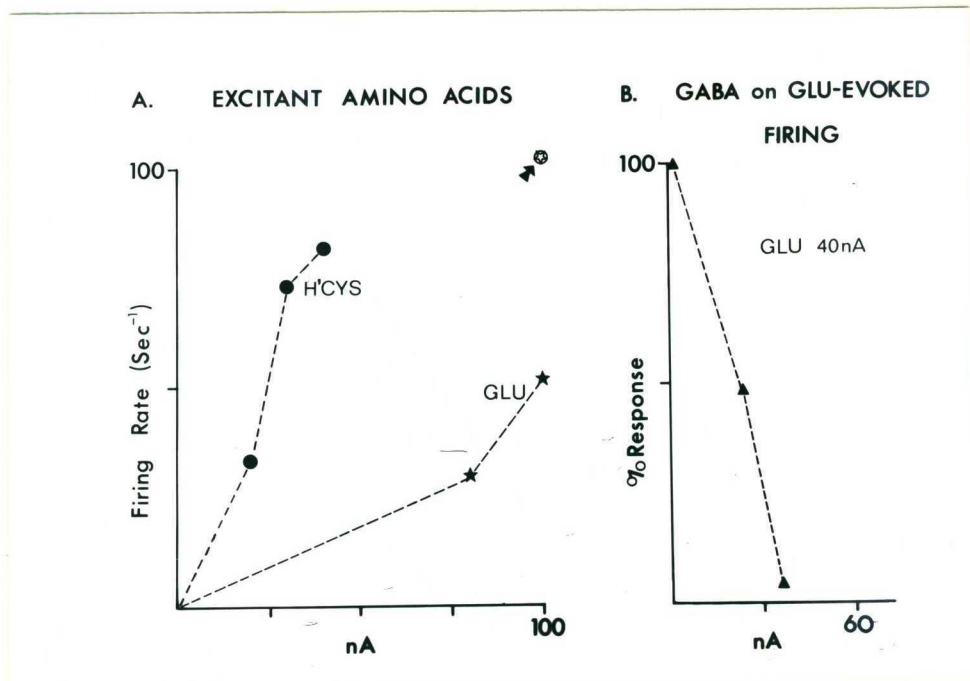


Fig. 7: Effect of amino acids on rubral activity. Explanations in text.

threshold for block did not affect axon conduction or change appreciably the axon membrane time constant. Experiments with reduced extracellular potassium concentration indicated a common mechanism for the inhibitory transmitter and for GABA which, it appeared, was destroyed by an enzymic process in the synaptic region.

Studies on spinal neurones (Curtis et al., 1959) in which intracellular recording of responses during GABA application was achieved, indicated that the compound causes an increase in resting membrane conductance, without any change in membrane potential, in contrast with recorded inhibitory potentials. This was accepted as indicating

that GABA has a 'non-specific depressant action' upon the neurone and that it is probably not a specific inhibitory transmitter in the mammalian nervous system.

3. The Biogenic Amines

i) Acetylcholine and related compounds

Histochemical examinations show only slight cholinacetylase activity in brachium conjunctivum and red nucleus (Feldberg and Vogt, 1948) and sparse, light cholinesterase staining in a few large cells of the red nucleus (Koelle, 1954).

a) Anaesthetized Preparation

Acetylcholine and carbachol, applied with currents of 60-100nA for periods of up to two minutes were found to have no effect on the synaptically evoked or amino acid-induced firing of rubral neurones in the anaesthetized preparation: acetylcholine was applied to eight cells from three different electrodes whilst carbachol was applied to two cells from one electrode.

b) Decerebrate Animal

Acetylcholine was applied to eight rubral neurones without effect on 'spontaneous' firing or the synaptic discharge following a single brachium conjunctivum volley.

The cholinergic blocking agent, dihydro-B-erythroidine failed to depress orthodromically evoked activity in the five cells examined and was without effect on spontaneous firing in the decerebrate preparation.

ii) 5-Hydroxytryptamine

a) Anaesthetized Preparation

In the anaesthetized cat, application of 5-HT failed to elicit

any apparent changes in the excitability of the nine rubral cells tested. The quiescent cells could not be made to discharge and the drug neither augmented nor depressed amino acid-evoked activity and the response to orthodromic stimulation appeared unaffected.

b) Decerebrate Cat

Results obtained by the use of the unanaesthetized preparation have been useful in indicating a probable difference between the modes of action of 5-HT and the phenylethylamines. 5-Hydroxytryptamine was applied to fourteen rubral cells with currents of between 10 and 100nA for durations of 30-200 seconds and a marked depressant effect was observed on the synaptic mechanisms.

The extracellular response recorded in the red nucleus is shown in figs. 8A and 9A. In fig. 8A after the initial negativity marking the stimulus artifact is the positive-negative waveform (b) indicating the arrival of the brachium volley in the red nucleus, then the negative wave (r) marking the rubral discharge after a synaptic delay.

Figures 8B and 9E, F demonstrate the depressant effect of 5-HT on the synaptic mechanism as seen in the decerebrate preparation. The cell from which the responses of fig. 8 were recorded was more susceptible to the influence of the indolealkylamine than that of fig. 9 and as seen in fig. 8B transmission was blocked by 5-HT with a current of only 40nA. In fig. 9E the latency of the postsynaptic activity is clearly increased by application of 80nA 5-HT by comparison with the controls (fig. 9A, D) and in fig. 9F 100nA has blocked response to brachial stimulation.

To establish whether or not the current applications were affecting neuronal firing regardless of any action of the drug, sodium ions (presumed 'inert' to the resting membrane which is assumed to

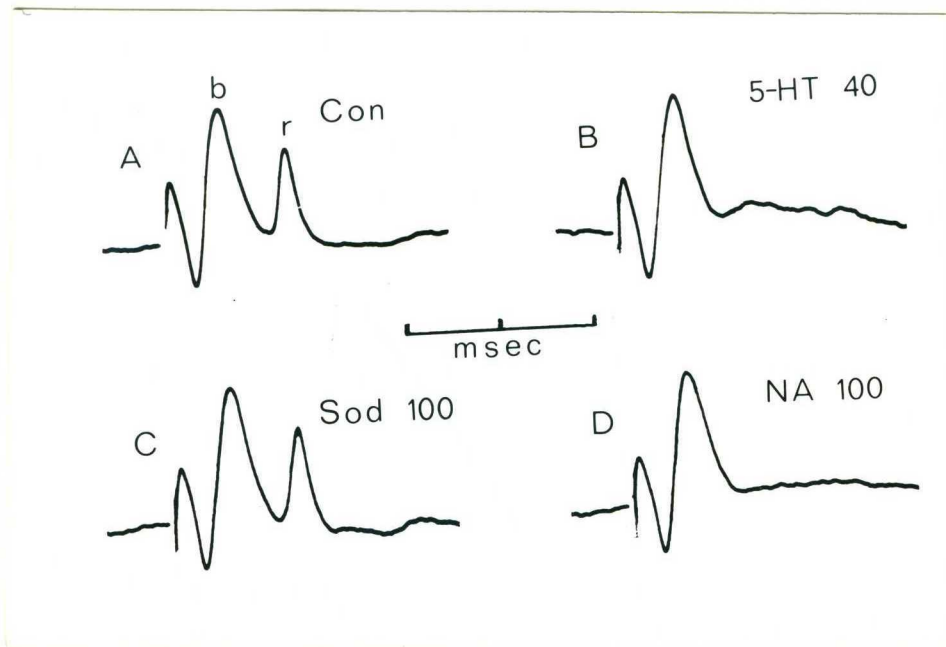


Fig. 8: See description in text. Numerals above the traces indicate the currents used to ionophorese the drugs.

have a low sodium conductance) were passed using currents of up to 100nA. If the sodium current depressed cellular excitability in a parallel manner to drug application, such results would have been deleted from the overall assessment. Hence, figures 8C, 9D, H, 10 (Sod. 60nA) and 11 (Sodium) are regarded as 'controls' along with those marked 'Con' which were taken from recordings of cells firing without extraneous currents being applied.

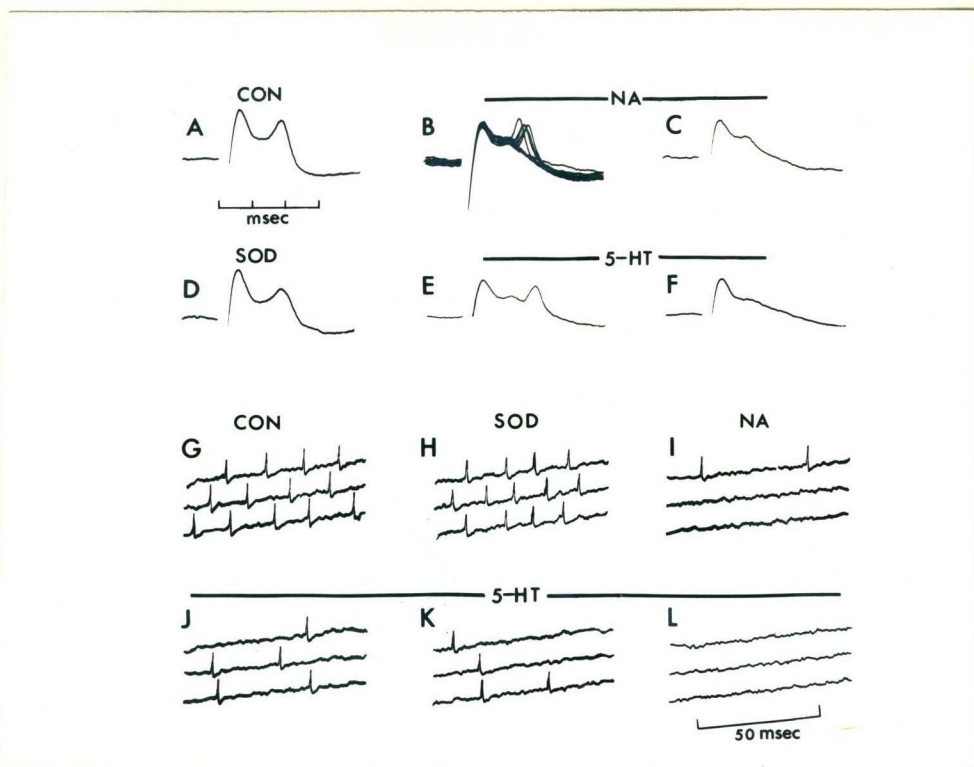


Fig. 9: Effect of noradrenaline and 5-hydroxytryptamine on rubral activity in the unanaesthetized cat.

A-F: The effect of the compounds on evoked activity

G-L: Depressant effect on spontaneous activity

The figure is described in the text.

Figures 9J-L and 10 (5-HT 50nA) illustrate the effect of the compound on two of the ten cells 'spontaneously' firing. When 5-HT was applied the firing rate decreased in six of the cells; in the majority, as depicted in fig. 10, the degree of depression appeared proportional to the current used and therefore the amount of drug applied until the current reached 40nA, which usually gave a near-maximal effect. This also appeared true when brachium conjunctivum volleys were used to elicit rubral activity: 40 nA 5-HT (as in fig. 8B) usually resulted in a

near-maximal transmission failure. The cell whose responses are shown in fig. 9 was an exception. As can be seen in fig. 9 J-L increasing the current (40nA, 80nA, 100nA) resulted in an increasing degree of depression until firing was abolished. The effect, on this autogenic discharge, of varying the iontophoresis current over a wide range is demonstrated in fig. 11, horizontal lines indicating the current being used, those below (positive to) the zero mark indicating 'ejecting current' and those above, 'backing off'. The vertical lines each represent the mean firing rate for the preceding 5 seconds, represented by the spaces between the lines. As can be seen by the rapid random firing persisting during sodium application, 5-HT was strongly depressant. Augmentation of spontaneous firing with homocysteate also appeared to be depressed (see Discussion).

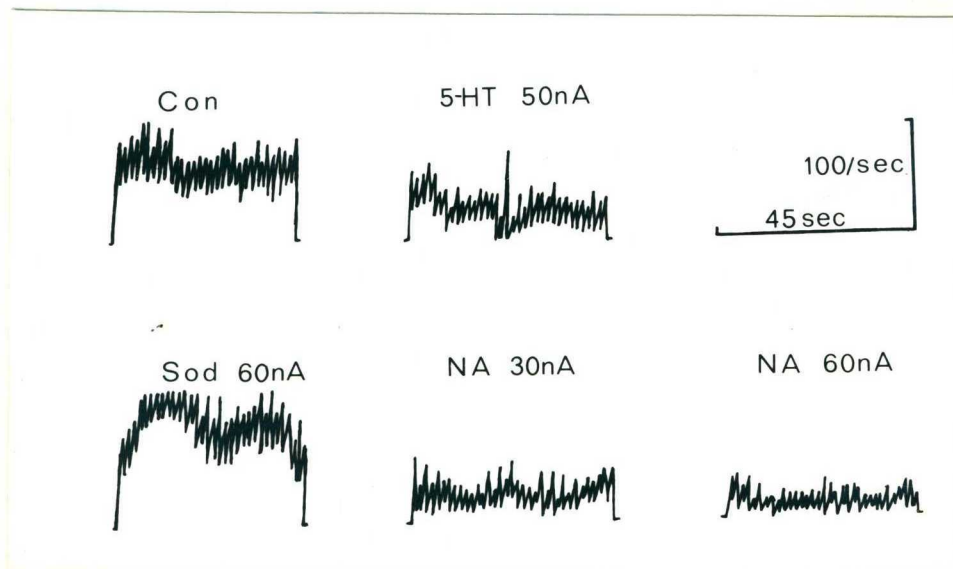


Fig. 10: Tracings of penwriter records. Effect of drugs on spontaneous rubral activity; described in the text. Note that sodium current seems to increase the firing rate by comparison with the controls.

iii) Dopamine, Noradrenaline and Adrenaline

a) Anaesthetized Animal

In the barbiturate anaesthetized cat, the effects of dopamine and of noradrenaline on synaptically evoked discharge were tested using sixteen cells with the former and twenty six cells with the latter. In none of these examinations did the amines block the rubral response. When these cells were excited by application of L-glutamate or DL-homocysteate, however, the amino acid-evoked activity was consistently depressed over a wide range of current strengths used and an excitatory effect was never observed (Cf. the effect of large adrenaline currents on cortical cells, Krnjević and Phillis, 1963).

In the chloralose anaesthetized animal synaptic firing was blocked in one cell only and by both amines, using a relatively high current of 100nA in the ejection of each (fig. 12A, B, E). The effect was observed only after the applications had proceeded for about 45 seconds and in each case recovery took 10-15 seconds after the drug current had been turned off. To establish that it was not the effect of passing increasing amounts of current, sodium ions were applied with the same current for a similar length of time: they did not affect the synaptic response (fig. 12D). As with the barbiturate anaesthetized cat amino acid-evoked firing was depressed. Fig. 12G shows the activity of the same cell as in fig. 12A-E being produced by applications of homocysteate ion with a current of 20nA. Continuous ejection of the phenylethylamines during a series of brief ('ON'-'OFF') stimulations with amino acid resulted in the activity being depressed; fig. 12H, I demonstrate the effect of 80 and 100nA of noradrenaline respectively. While dopamine (fig. 12K) 100nA acts similarly, the effect observed here is not as marked as with noradrenaline. As in the other investigations sodium (fig. 12J) 100nA was passed as a control: had it affected the discharge rate, any results from the cell would have been deleted.

The decrease in discharge rate of rubral neurones excited by amino acids was found to be proportional to the amount of current used to produce the applications of phenylethylamine (fig. 13).

Adrenaline was tested on nine cells in the anaesthetized animal and its effect on amino acid evoked firing was comparable to that of noradrenaline and dopamine. The electrode containing noradrenaline and dopamine which was used in the prevention of synaptic activation of the cell in fig. 12 did not contain adrenaline, so no indication was obtained of its relative ability to block synaptically evoked firing.

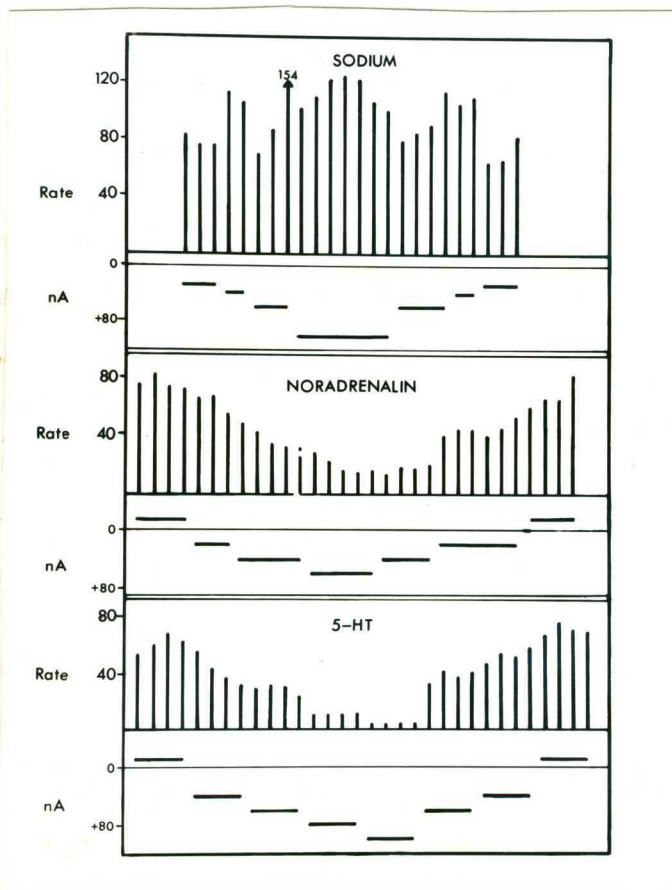


Fig. 11: Effect of varying drug currents on spontaneous rubral activity. The maximum current used (horizontal lines) was 100nA. Currents above the zero line indicate backing currents and were decided by the 0.5V backing potential and the tip resistances of the barrels.

During 'backing off' tartrate was being ejected from the noradrenaline barrel and sulphate from the 5-HT. Sodium was delivered from an amino acid barrel. The results are described in the text.

b) Decerebrate Cat

In the decerebrate, unanaesthetized animal the results were very similar.

Figure 9B shows a threshold condition of a brachio-rubral synapse being activated by a brachium conjunctivum volley while under the influence of an application of noradrenaline (80nA): the latency of the discharge varied considerably and was generally longer than in the controls (fig. 9A, D) and at times the transmission was blocked. Figure 9C illustrates the synaptic response being blocked with an application of noradrenaline (100nA), as does fig. 8D.

The spontaneous firing was consistently depressed by noradrenaline, even when very small currents were used (e.g. 10nA) and was blocked by larger currents (figs. 9I, 11). Depression of spontaneous firing appeared proportional to the current used: this progressive failure is illustrated by figs. 10 (noradrenaline 30nA, noradrenaline 60nA) and 11.

3. Biologically Active Phenylethylamines

The finding that the biogenic phenylethylamines depressed neuronal excitability prompted the testing of a selected group of biologically active derivatives, amphetamine, isoprenaline and 4-methoxyphenylethylamine. Amphetamine was chosen on the basis of its psychomotor stimulant activity and its ability to facilitate mono- and polysynaptic activity in the spinal cord (Killam, 1959); isoprenaline, for its

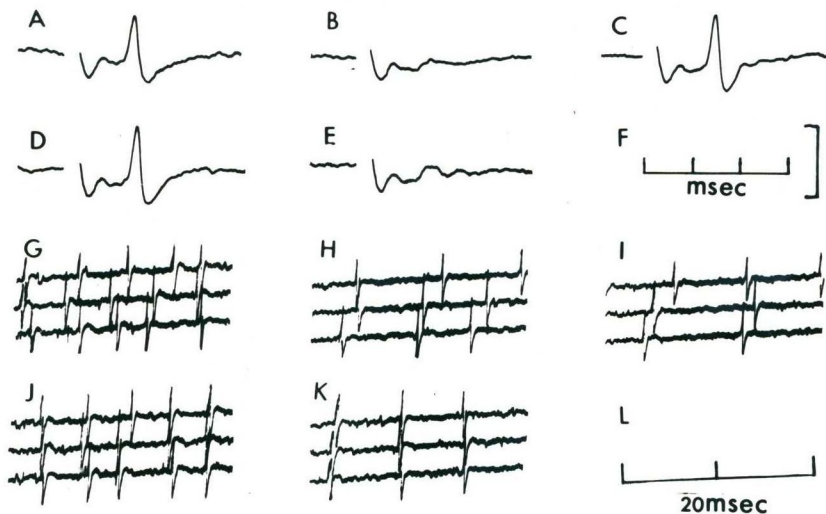


Fig. 12: Effect of catecholamines on rubral activity in the chloralose anaesthetized cat.

A-E: Responses in red nucleus subjected to brachial volleys

G-K: Activity of rubral cells excited by DL-homocysteate

A, C, G: Control records (no current)

D, J: Current controls (sodium 100nA)

B: Noradrenaline, 100nA; E: dopamine, 100nA

H, I: Noradrenaline, 80 and 100nA respectively

K: Dopamine 100nA

Vertical calibration (F), 400uV (refers to A-E)

sympathomimetic properties and 4-methoxyphenylethylamine (Roche 1-2070/1) which induces rigidity in cat and monkey (Ernst, 1962 a, b).

Only a small number of cells was tested and only in the barbiturate anaesthetized preparation. Synaptic firing was not affected but depression of amino acid-induced firing, in a manner similar to that caused by the biogenic phenylethylamines, was observed.

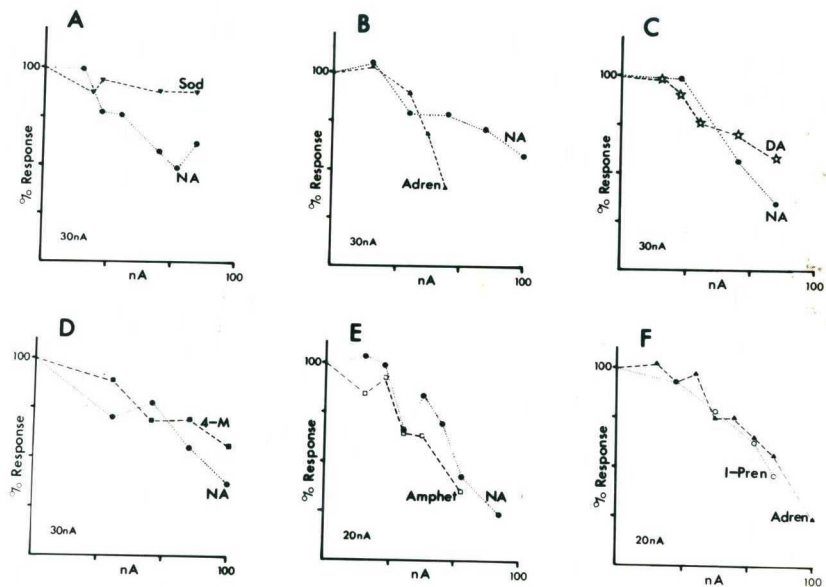


Fig. 13: Depression of amino acid-evoked firing by biologically active phenylethylamines. Current values shown in lower left-hand corner of each pair of arcs indicates current used to ionphorese L-glutamate. Sodium control in A. Barbiturate anaesthetized preparation. Sod.: Sodium, NA: noradrenaline, Adren: adrenaline, DA: dopamine 4-M: 4-methoxyphenylethylamine, Amphet: amphetamine, I-Pren: isoprenaline.

Because, in any one solution used in these experiments, the transport numbers of the ions present were not known, because the electrical properties of one ionic solution differ from those of another and because there are other physical factors determining flux rates from microelectrodes, accurate assessment of the number of ions

issuing from a barrel under experimental conditions cannot be made by monitoring the current flowing in the electrophoresis circuit alone. Theoretical calculations have been presented and supported experimentally (Krnjević et al., 1963) showing that physical dissimilarities, not readily detectable, between electrode barrels are important factors in determining flux rates. Using one salt (acetylcholine chloride) gross variations in the amount of active substance effluxing from different barrels during electrophoresis by a given current were observed: however, for a given barrel, the amount of drug passed is approximately linearly related to the current. Because of this limitation it is invalid to present a potency scale for substances such as these phenylethylamines unless comparisons are repeated a sufficient number of times to provide a statistically significant result. No potency estimates have been offered as a result of these experiments, but there does not seem to be a significant difference between the effectiveness of any two compounds used (see fig. 13).

DISCUSSION

The local application of 5-hydroxytryptamine and certain derivatives of phenylethylamine, among them the endogenous catecholamines, to rubral neurones has resulted in the prevention of these cells from responding to volleys in the brachium conjunctivum and to spontaneous input, inferred, from the results of Calma (1952) and Massion and Albe-Fessard (1963) to be from discharges in the brachium.

The results cited in this thesis, which have been obtained using cats in two different states, have provided evidence for different mechanisms of action of 5-HT and the phenylethylamines in exerting depressant effects on the brachiorubral synapse system.

The influence of anaesthetic agents on the physiological state of the nerve cell is little known. Recent experiments (Løyning et al., 1964) in which blood pressure and oxygen tension in the spinal cord have been monitored have given results indicating that the basic mechanism of barbiturate-block of synaptic transmission at the motoneurone is manifest in a reduction in the size of the action potential invading the presynaptic terminals and thus a reduction in transmitter output, although close examination revealed no reduction in the excitability of afferent nerve terminals under the influence of the barbiturate, by comparison with the controls. These results were at variance with those of Somjen and Gill (1963) who used two dissimilar 'general anaesthetics' in a study on motoneurones. The latter workers found that under the influence of thiopental and diethyl ether the rate of rise of the e.p.s.p. and its amplitude were reduced, without any delay in its onset or alteration of the resting potential, and the threshold for spike production in the motoneurone was increased. The experiments of Løyning et al. indicated that large doses of barbiturate (they used thiamylal sodium) depressed pO_2 and blood pressure, which may have caused a toxic disturbance of metabolism and a reduction in metabolism due to hypoxia and which may

have contributed to the results of the earlier experiments, but which were, in fact, shown to be unrelated to the primary mechanism of transmission block. When small doses of thiamylal sodium were administered there was a reduction in the monosynaptic reflex which was not subtended by pathological environmental changes. Intracellular recording from motoneurons of the spinal cord revealed that the monosynaptic e.p.s.p. was decreased by thiamylal. The firing threshold of the motoneurons was not altered, whether the spike potential was elicited synaptically or by passage of rectangular direct current pulses. The spike potential, resting potential, electrical properties and accommodation of the motoneurons were unaltered during thiamylal anaesthesia. Results attributable to the anaesthetic effect have been observed on the brachiorubral synapse system: the synaptic latency is clearly increased and activity present in the unanaesthetized, decerebrate animal is depressed when the cat is anaesthetized with pentobarbital. Whether chloralose has the same action on the synaptic mechanism has not been investigated, but a difference between its mode of action and that of the barbiturates can be inferred from the results of Massion and Albe-Fessard (1963).

In the lateral geniculate nucleus amino acid-evoked firing was resistant to depression by 5-HT (Curtis and Davis, 1962) whereas synaptic transmission was clearly blocked. Similar results have been obtained in the red nucleus, where 5-HT was without effect on the activity evoked by amino acids in the barbiturate anaesthetized animal, but where synaptically evoked activity was blocked and the spontaneous activity seen in the decerebrate preparation was markedly depressed. Antidromic action potentials set up in geniculate fibres did not fail to invade the somadendritic membrane (Curtis and Davis, 1962) and there was no alteration of the conformation of the extracellularly recorded spike suggestive of an alteration of postsynaptic neurone membrane properties. As a result of this study the authors concluded that 5-hydroxytryptamine probably has a presynaptic site of action, interfering with the release of transmitter from the presynaptic terminals in some way or, alternatively, by

competing at the subsynaptic membrane 'receptor site' with the excitatory transmitter. If the former alternative were the case, it is most probable that the mechanism would involve some form of membrane reinforcement, depressing the vesicular (or other secretory inclusion) disruption, or other neurohormone-releasing action, rather than reducing the amplitude of the terminal-invading action potential by some means, since the size of the presynaptic field was unaltered by the drug (see Curtis and Davis, 1962, fig. 2): Løynning et al. (1964) recognized the negative-peak amplitude of the presynaptic field response to be an index of action potential amplitude. The present results are compatible with either of the alternatives proposed by Curtis and Davis and are supportive of the contention of the previous workers that the action is not directed at the properties of the postsynaptic electrically excitable membrane. The observation that amino acid augmentation of low levels of spontaneous activity was reduced by 5-HT is explainable on the grounds that the synaptic bias would be removed or reduced by the drug, leaving the amino acid effect 'bare'.

From a study of the locomotor activity of mice treated with various compounds affecting catecholamine metabolism, Rossum and Hurkmans (1964) have classified psychomotor stimulant drugs in four groups which they maintain cause psychomotor excitation by different mechanisms. Amphetamine and closely related compounds are thought to interact directly with receptors for catecholamines in the brain, being rapidly effective and not dependent on the integrity of the central catecholamine stores. The response to cocaine was totally dependent on the presence of intact tissue stores of dopamine, further evidence supporting the suggestion of Barbeau and Sourkes (1961, see General Introduction) that dopamine is important in the function of the basal ganglia.

The results cited in this thesis are also indicative of phenylethylamines all acting at the same site, whether or not this site is a normal central catecholamine 'receptor'.

The result which indicates a probable modal difference between the phenylethylamines and the indole compound is the depression of amino acid-evoked firing by the former in the barbiturate anaesthetized cat. This result may be interpreted in several ways. The phenylethylamines could cause a decrease in the sensitivity of the ionic conductance mechanisms of electrically excitable membranes to depolarization, thus increasing threshold and reducing the size of the spike, as has been observed for procaine and cocaine in the voltage-clamped squid axon (Shanes et al., 1959): Curtis and Phillis (1960) illustrated the effect for procaine on the motoneurone. Alternatively, low impedance 'patches' could be induced in parts of the membrane such that excitatory potentials would be 'short-circuited', and other 'non-specific' actions at the membrane are possible. These are the mechanisms which have been suggested as unlikely for 5-HT. This would explain their ability to block amino acid-induced firing as well as that elicited by volleys in the brachium conjunctivum and by the 'spontaneous discharge mechanism'. These compounds may also have an action on the components of the synapse system, as has been suggested for 5-HT, above. Extracellular recording in the olfactory bulb of the rabbit during application of the amines (von Baumgarten et al., 1964) failed to reveal any alteration in the characteristics of the antidromically evoked spike of mitral cells and they concluded that the compounds were not exerting any influence on conductance mechanisms of the electrically excitable membrane. Unfortunately, as examination of the published figures of these authors reveals, the recorded antidromic activities were made up of field responses from groups of neurones firing slightly out of phase with one another. This

asynchrony would serve to disguise small changes in the 'picture' of the field: the blocking of an action potential in one or a number of fibres in the recording field of the electrode would result in a reduction of the amplitude of the field response. That this was not observed could be explained on the grounds that the concentration of amine produced in the extracellular environment, while sufficient to block orthodromic transmission, may be insufficient to block the propagation of an action potential initiated in an unaffected part of the fibre. Such a state of affairs would be possible in certain mechanistic circumstances, such as will now be described. From observations of several workers (see especially Araki and Terzuolo, 1962), it has been documented that the threshold for regenerative activity in soma-dendritic membrane is considerably higher than in initial segment and other axonic membrane. The electrotonic depolarization of the soma-dendritic membrane by the synaptic mechanism is insufficient to initiate an action potential in this area of membrane and it only arises by invasion of the soma by an action potential initiated in the initial segment, which is then refractory, so that the soma-dendritic spike does not propagate back along the axon. Evidence of this is seen in the records shown in the Results. The waveform of the rubral discharge, where this has been elicited by brachial input (see 'evoked' and 'spontaneous' records), has only a small late positive component (probably due to the spike propagating peripherally in the dendrites) by comparison with that of the amino acid-evoked response. The chemically elicited activity arises in the membrane under the electrode and propagates away from the initiation-recording site, as evidenced by a large late positive component. If the drugs were to reduce somatic membrane impedance the length constant of the membrane would be reduced, the depolarization of the initial segment in response to synaptic input would be reduced, possibly below threshold, and consequently the excitatory conduction would be blocked. However, an

antidromic action potential may still be able to invade the soma and unless the resolution afforded by the recording technique was sufficient to reveal changes in the spike waveform, such a test for a postsynaptic site of action would give a misleading result.

Curtis[†] and Davis[†] (1962) interpretation of the result for 5-HT is, however, acceptable because of the failure of the compound to block amino acid-induced discharge. One would expect that if the excitability of the postsynaptic membrane had been depressed, amino acid firing would have been reduced as it was by the phenylethylamines. Examination of filmed 'spontaneous' activity under the low power objective of the microscope revealed a very small positive-negative waveform preceding the rubral spike which was probably due to the presynaptic activity. Examination of fig. 9G-L gives an indication of the even periodicity of the 'spontaneous' discharge. If a transients-analysis system of sufficient resolution were available the recognition of presynaptic activity in such records could be made more positively and abortive presynaptic activity could be distinguished from the baseline noise. Reduction in the frequency of presynaptic firing during the action of a drug would be positively indicative of a presynaptic site of action, but the failure to reduce presynaptic activity would leave the situation as it was left by Curtis and Davis, for 5-HT, after their study of the lateral geniculate.

The inadequacy of the resolution generally afforded by the extracellular recording technique limits an analysis of the membrane phenomena as may be expected to occur under the experimental conditions as described: the drugs examined may even have exerted an effect on the neurones manifest as a depolarization or hyperpolarization, but the extracellular situation of the electrode, together with the recording through a condenser-coupled amplifier, would not allow the slow change in membrane potential concomitant with the slow build-up of applied

drug to be recorded. Increased acuity in analytical procedures would be afforded by the use of intracellular recording: it was only by the use of intracellular recording techniques that Ld'ynning et al. (1964) were able to show, unequivocally, that the fundamental blockage of synaptic transmission by barbiturate occurs elsewhere than in the postsynaptic member. These authors did not indicate what proportion of the motoneurone population examined responded to ortho- or antidromic stimulation with e.p.s.ps or fully developed action potentials: during the period in which the present pharmacological studies were carried out, attempts were made to record intracellular potentials from rubral cells with KCl or K_2SO_4 -filled glass microelectrodes. In spite of the fact that extracellular recording indicated extensive excitatory activity in the nucleus following brachial volleys, very few of the large number of neurones impaled gave sign of responsiveness and these could not be studied for very long periods. This experience has been indicative that tediousness and possibly fruitlessness could be the outcome of attempts to study pharmacological problems of the red nucleus with this method.

Ideally, the size of the rubral neurone is suitable for intracellular examination (some diameter, about 50u) and one may cite the use of concentric electrodes as the basic tool for a definitive study of neuropharmacological phenomena at this site. These electrodes consist of a fine central barrel filled with a suitable low-impedance solution (e.g. KCl, K_2SO_4 , Kcit.) for intracellular recording, jacketed by another drawn glass tube with a tip of 10-12u diameter, prepared as were the multibarrel pipettes used in the present study (see Methods) and filled with a solution of the drug to be iontophoresed. This outer barrel is fixed with its tip 40-50u from the tip of the fine inner barrel, so that while the latter is within the cell, recording, drug can be applied to the external environment. By the use of a suitable bridge arrangement,

by which tip potentials which arise as artifacts can be "bucked", or compensated for (e.g., see that of Martin and Pilar, 1963a), current may be passed into the cell via the recording electrode in order to more rigorously study changes, if any, in the membrane conductance.

If it were shown that the phenylethylamines do not have an action at the electrically excitable postsynaptic membrane, the results with the amino acids would require that the phenylethylamines be competitors of the mono-amino dicarboxylic acids at their site of action on the membrane. Of course, a combination of these suggested mechanisms would not be impossible.

During the course of the experiments it was thought that the failure of the synaptically evoked response during phenylethylamine application in the chloralose anaesthetized animal may have been fortuitous: the result could have been explained by assuming that the synaptic input to the rubral cell was just at threshold and the cell was failing randomly, coincident with the administration of the drugs. However, the succeeding experiments in the unanaesthetized cats confirmed the ability of the phenylethylamines and 5-hydroxytryptamine to block the brachiorubral synapse, so that it would now appear that our inability to block the synaptic mechanism in the barbiturate anaesthetized animal may have been due to chance. Stimulation of the brachium conjunctivum with high-voltage pulses from bipolar electrodes, as was employed in this study, results in an extremely powerful input to the red nucleus cells. Histological investigations (Cajal, 1911) have shown rubral cells to have diffuse and extensive dendritic arborizations: Cajal described them as "extending almost the width of the nucleus, which is approximately 1-1.5 mm". The observations indicate that one rubral cell receives synaptic input from a large number of brachial fibres and that activation of many, or all, of these inputs using our method of stimulation is possible, but would be a physiological rarity. The inability to block

activity evoked by brachial stimulation in the majority of cells tested, notably those in the barbiturate anaesthetized animal which were not blocked at all, could be due to the iontophoretically released drugs exerting an effect which is insignificant by comparison with the powerful synaptic drive. Equally as plausible, of course, is the possibility that pentobarbital is antagonistic to the compounds tested. If this antagonism were operative, it would necessarily be exerted on some part of the synaptic mechanism and not on that part of the membrane where the amino acid 'receptors' are, since these are still sensitive to phenylethylamines.

What is the significance of these observations? Reviewing the literature it can be seen that 5-HT and the phenylethylamines have predominantly depressant actions on the neuronal systems studied, although in a number of areas facilitation has also been seen. A duality of response is not confined to these compounds; acetylcholine exerts mixed effects on certain systems. It was seen to have an initial depressant effect on neurones of the ventrobasal (Andersen and Curtis, 1964) and ventrolateral (Davis et al., 1964) thalamic nuclei, preceding the excitatory action which has led these workers to be aware of the possible synaptic function of this amine in these areas. Andersen and Curtis (1964a) suggested that cholinceptive inhibitory synapses may be present, but it seems equally possible that the amine may exert some 'non-specific' action on membranes. There is, generally, very little evidence positively indicating a synaptic role for these endogenous compounds and it is questionable whether neuropharmacologists have been able to do more than document findings of possibly doubtful or at least, as yet, unknown significance. While the preceding evidence may be thought to favour the view that 5-hydroxytryptamine and the catecholamines are rubral neurohormones, it by no means adds up to rigorous proof. As has been implied in this discussion, it is just as likely that the actions which we have observed,

attributable to the amines, were exerted on a portion of the neurone and in a manner which was totally unrelated to the synaptic function, if such exists. If the amines are present as transmitters it is improbable that they are released from brachial fibres: there is every indication that the brachium exerts a purely excitatory input to the red nucleus and this cannot be duplicated by the compounds. The red nucleus receives several other inputs, possibly including trigeminal projections from tactile receptors (Eisenman et al., 1964) and only by a study of the interactions of these inputs at the red nucleus could synapses be characterized.

It is not impossible that if the amines are present in varicose fibres, as have been observed elsewhere, that their function here is to exert a non-specific depressant effect on the whole nucleus, subject to secretion from those fibres which would be activated by some input, the function of which would be to control the 'tone' or 'excitability level' of the nucleus. This is made the more plausible by Burnstock's suggestion (in seminar, Pharmacology Department, University of Sydney, 1964) that in smooth muscle beds each adrenergic fibre varicosity may be equivalent to a presynaptic terminal and may release transmitter when the fibre is invaded by an action potential.

In conclusion, then, it can be said that in spite of the localization of biogenic amines in presynaptic fibres in the central nervous system morphologically similar to the terminal fibres of peripheral sympathetic nerves and the separation of organelles containing the amines from disrupted nerve ending particles in brain homogenates by differential centrifugation techniques, there is no conclusive evidence that these amines are involved in synaptic transmission. Is their presence, in fact, indicative of transmission at some specialized synapse, or is it conceivable that while not involved directly in synaptic transmission, non-synaptic chemical activity may still be physiologically important?

APPENDIX IA Derivation of the 'Goldman Equation' from the Kinetic Theory

This derivation has been presented by Dr. E. P. George and is similar in form to that presented by Hodgkin and Katz (1949). For the most rigorous, original derivation from first principles of expressions for membrane potential, impedance and conductance, see Goldman (1943).

The basic assumptions are (i) that ions in the membrane move under the influence of diffusion and the electric field in a manner which is essentially similar to that in free solution, (ii) that the electric field may be regarded as constant throughout the membrane, (iii) that the concentrations of ions at the edges of the membrane are directly proportional to those in the aqueous solutions bounding the membrane and (iv) that the membrane is homogeneous. While some of these assumptions, particularly the latter, may be invalid the applicability of the Goldman equation and therefore of these assumptions has been demonstrated on biological systems.

Consider a cylindrical portion of membrane of unit cross-sectional area:

Let n = number of ions/unit volume at x

$n + dn$ = number of ions/unit volume at $x + dx$

where x is a plane perpendicular to the long axis of the cylinder and $x + dx$ is another plane an infinitesimal distance from the first and parallel to it. The pressure, P , due to these ions is

$$\text{at } x \quad P = 1/3 (m \cdot n \cdot \bar{c}^2) \quad (1)$$

$$\text{at } x + dx, P + dP = 1/3 (m \cdot (n + dn) \cdot \bar{c}^2) \quad (2)$$

$$\text{Pressure difference, } dP = 1/3 (m \cdot \bar{c}^2 \cdot dn) \quad (3)$$

This force acts on $n \cdot dx$ ions

$$\therefore \text{Force per ion} = \frac{-dP}{n \cdot dx} = \frac{-m \cdot \bar{c}^2 \cdot dn}{3n \cdot dx} \quad (4)$$

(negative sign because force acts from right to left in diagram, in which positive direction is, by convention, left to right).

$$\text{But } m \cdot \bar{c}^2 = 3k \cdot T \quad (5)$$

where k = Boltzmann's constant

and T = absolute temperature

$$\therefore \text{force/ion} = \frac{-k \cdot T \cdot dn}{n \cdot dx} \quad (6)$$

Let v = drift velocity

and ρ = medium resistance ($= \frac{F}{v}$, cf. Ohm's law)

$$\text{defined by } \rho = \frac{\text{force/ion}}{v} \quad (7)$$

If u = ionic mobility = velocity of ion in unit field

and ξ = ionic charge

$$\rho = \frac{\xi}{u} \quad (8)$$

$\therefore$ from (7) and (8)

$$\rho = \frac{\xi}{u} = \frac{\text{force/ion}}{v}$$

$$\begin{aligned} \text{and } v &= (\text{force/ion}) \cdot \frac{u}{\xi} \\ &= \frac{-k \cdot T \cdot u \cdot dn}{n \cdot \xi \cdot dx} \end{aligned} \quad (9)$$

Current due to diffusing ions,

$$\begin{aligned} I_d &= n \cdot \xi \cdot v \\ &= \frac{-k \cdot T \cdot dn \cdot u}{dx} \end{aligned} \quad (10)$$

If an electric field of strength E is present, ionic current in the field,

$$I_f = n \cdot u \cdot E$$

But $E = \frac{-dV}{dx}$ (usual convention for sign of V)

$$= \frac{V}{a} \quad (\text{constant field assumption})$$

∴ Total ionic current,

$$I = \frac{-k \cdot T \cdot u \cdot dn}{dx} + \frac{n \cdot u \cdot \xi \cdot V}{a} \quad (11)$$

Rearranging (11)

$$\frac{dn}{dx} - \frac{n \cdot \xi \cdot V}{k \cdot a \cdot T} = - \frac{I}{k \cdot T \cdot u} \quad (12)$$

Left hand side is recognizable as part of the differential coefficient of a product,

e.g. $\frac{d(n \cdot w)}{dx} = w \cdot \frac{dn}{dx} + n \cdot \frac{dw}{dx}$

ut $\frac{dw}{dx} \cdot \frac{1}{w} = - \frac{\xi \cdot V}{k \cdot a \cdot T}$

$$\therefore \frac{dw}{dx} = - \frac{\xi \cdot V}{k \cdot a \cdot T} \cdot w$$

$$\therefore w = \exp(-\xi \cdot V \cdot x / k \cdot a \cdot T)$$

$$\therefore \frac{d(n \cdot \exp(-\xi \cdot V \cdot x / k \cdot a \cdot T))}{dx} = - \frac{I}{k \cdot T \cdot u} \cdot \exp(-\xi \cdot V \cdot x / k \cdot a \cdot T)$$

Multiply throughout by dx and integrate

$$\int d(n \cdot \exp(-\xi \cdot V \cdot x / k \cdot a \cdot T)) = \int - \frac{I}{k \cdot T \cdot u} \cdot \exp(-\xi \cdot V \cdot x / k \cdot a \cdot T) \cdot dx$$

$$\therefore n \cdot \exp(-\xi \cdot V \cdot x / k \cdot a \cdot T) = \frac{I \cdot a}{\xi \cdot V \cdot u} \cdot \exp(-\xi \cdot V \cdot x / k \cdot a \cdot T) + \text{const} \dots (13)$$

For the membrane it has been assumed that

$$n_o = B [n]_1$$

$$n_a = B [n]_o$$

where B is the partition coefficient in question; i.e. density in membrane at inner boundary, $n_o = B [n]_i$ where $[n]_i$ is the concentration in the inside solution; similarly for the external boundary.

Applying boundary conditions to find the constant of integration in (13)

when $x = 0$,

$$n = B [n]_i$$

$$B[n]_i = \frac{I \cdot a}{\xi \cdot V \cdot u} + \text{const.}$$

$$\therefore \text{const.} = B[n]_i - \frac{I \cdot a}{\xi \cdot V \cdot u}$$

$\therefore$ (13) becomes

$$n \cdot \exp(-\xi \cdot V \cdot x / k \cdot a \cdot T) = \frac{I \cdot a}{\xi \cdot V \cdot u} \cdot \exp(-\xi \cdot V \cdot x / k \cdot a \cdot T) + B[n]_i - \frac{I \cdot a}{\xi \cdot V \cdot u} \quad (14)$$

Multiply throughout by $\exp(\xi \cdot V \cdot x / k \cdot a \cdot T)$

$$\text{then } n = \frac{I \cdot a}{\xi \cdot V \cdot u} + \left\{ B [n]_i - \frac{I \cdot a}{\xi \cdot V \cdot u} \right\} \exp(\xi \cdot V \cdot x / k \cdot a \cdot T) \quad (15)$$

At $x = a$, $n = B[n]_o$

$$\therefore n_a = B[n]_o = \frac{I \cdot a}{\xi \cdot V \cdot u} + \left\{ B [n]_i - \frac{I \cdot a}{\xi \cdot V \cdot u} \right\} \exp(\xi \cdot V / k \cdot T)$$

$$\therefore B[n]_o - B[n]_i \cdot \exp(\xi \cdot V / k \cdot T) = - \frac{I \cdot a}{\xi \cdot V \cdot u} (\exp(\xi \cdot V / k \cdot T) - 1) \quad (16)$$

$$\therefore I = \frac{B \cdot \xi \cdot u \cdot V}{a} \cdot \frac{[n]_o - [n]_i \cdot \exp(\xi \cdot V / k \cdot T)}{1 - \exp(\xi \cdot V / k \cdot T)} \quad (17)$$

Since $\frac{k \cdot T}{\xi} = \frac{R \cdot T}{F}$, write (17) viz:

$$I = \frac{B \cdot F \cdot u \cdot V}{a} \cdot \frac{[n]_o - [n]_i \cdot \exp(F \cdot V / R \cdot T)}{1 - \exp(F \cdot V / R \cdot T)}$$

where $[n]$'s are now in gram mols. per ml.

Hodgkin and Katz (1949) introduced the nomenclature:

$$\text{Permeability coefficient} = P = \frac{u \cdot B \cdot R \cdot T}{a \cdot F}$$

Substituting in (18)

$$I = \frac{P \cdot F^2 \cdot V}{R \cdot T} \cdot \frac{[n]_O - [n]_i \cdot \exp(F \cdot V / R \cdot T)}{1 - \exp(F \cdot V / R \cdot T)} \quad (19)$$

Consider a number of ionic species, e.g. K^+ , Cl^-

$$I_K = \frac{P_K \cdot F^2 \cdot V}{R \cdot T} \cdot \frac{[K^+]_O - [K^+]_i \cdot \exp(F \cdot V / R \cdot T)}{1 - \exp(F \cdot V / R \cdot T)} \quad (20)$$

$$I_{Cl} = \frac{P_{Cl} \cdot F^2 \cdot V}{R \cdot T} \cdot \frac{[Cl^-]_i - [Cl^-]_O \cdot \exp(F \cdot V / R \cdot T)}{1 - \exp(F \cdot V / R \cdot T)}$$

$$\text{i.e. } \sum_n I_n = \frac{F^2 \cdot V}{R \cdot T} \cdot \left\{ \frac{\sum_n P_n [n^+]_O - \sum_n P_n [n^+]_i \cdot \exp(F \cdot V / R \cdot T)}{1 - \exp(F \cdot V / R \cdot T)} \right\} \quad (21)$$

At equilibrium, $I = 0$

$$\text{and } \sum_n P_n [n^+]_O = \sum_n P_n [n^+]_i \cdot \exp(F \cdot V / R \cdot T)$$

$$\text{or } V = \frac{R \cdot T}{F} \cdot \ln \left\{ \frac{P_K [K^+]_O + P_{Na} [Na^+]_O + P_{Cl} [Cl^-]_i + \dots}{P_K [K^+]_i + P_{Na} [Na^+]_i + P_{Cl} [Cl^-]_O + \dots} \right\}$$

APPENDIX II

Regenerative Activity and Rectification

In electrically excitable membranes the transducer actions are evoked initially by an electrical stimulus and may be further modified as the membrane potential changes. Thus there may be regeneration. This regeneration may tend to increase the initial electrogenesis, to restore the resting state, or to increase the internal negativity beyond its resting value. The regenerative feature of electrically excitable activity is usually regarded as a property of depolarizing electrogenesis, but it may also be manifested by processes that exert repolarizing or hyperpolarizing electrogenic influence.

The most conspicuous and commonly recognized electrogenic activities are increases in membrane conductance which are initiated by depolarizing stimuli and themselves cause net additional depolarization. For example, in the squid giant axon, frog node of Ranvier and mammalian and amphibian striated muscle, the rising phase of the action potential is due to a 'snowballing' increase in sodium conductance. This increase in conductance on depolarization is termed rectification. In the normal action spike, as it is commonly observed, the falling phase is due to an increase in potassium conductance, which is slightly delayed in onset. This is termed delayed rectification, and coupled with the fall in sodium conductance which occurs after the initial rise and depolarization, helps repolarize the membrane. The fall in potassium conductance consequent to this repolarization is delayed by comparison with the sodium conductance inactivation and contributes to the after-hyperpolarization of the membrane.

Commonly, 'rectification' is an increase in conductance in depolarizing potential changes and 'anomalous rectification' is an increase in conductance consequent to hyperpolarization.

APPENDIX IIIMuscle Spindle Fibres and Their Innervation

Mammalian muscle spindles are small fibre groups which act as sensing organs relaying information to the cord and higher centres about the stretch-condition of the 'extrafusal' fibres via their afferent innervation. The spindles have their own motor innervation which sets their "sensing level" so that it is appropriate to the physiological requirement and by an afferent-efferent interaction exert a prominent influence over the performance of striated muscle.

The spindles consist of fibres of two types:

- i) Nuclear bag fibres: these fibres have large numbers of nuclei agglomerated in an enlargement of the fibre, situated near the middle of their long axes, which appears like a "bag".
- ii) Nuclear chain fibres: these fibres have their nuclei arranged in series, like a chain, in rather sparse population along the length of the fibres. There is no central enlargement.

Afferent innervation of muscle spindles may be broadly classified into two groups:

- i) Large diameter, high conduction-velocity fibres whose endings are situated in the centre of the long axes of the spindle fibre, wrapping around it, in a spiral configuration. These are group I or 'annulospiral' afferents.
- ii) Small diameter, low velocity fibres with endings near the poles (myotube region) of the muscle fibres. These are designated group II afferents. Group II endings, are spiral on nuclear chain intrafusal fibres, but more diffuse ('flower spray') on nuclear bag.

The motor innervation of the spindle fibres consists of small-diameter gamma motoneurones. These motoneurones end on end-plates situated in the myotube (polar) region of the nuclear bag fibres, and on end-plates more diffusely located on nuclear chain fibres. The simple nerve fibres innervating the single end-plates of nuclear bag fibres are designated γ_1 , and those with more complex endings on nuclear chain fibres, γ_2 .

Two types of activity have been recorded in afferent fibres from mammalian muscle spindles in response to stretch. Secondary endings (Group II) characteristically fire at a rate proportional to the instantaneous length of the muscle fibre while primary endings (Group I) fire at a rate which is affected by the rate of stretch, so that they signal both the instantaneous length of the muscle and the velocity at which it is being stretched. The latter signal is referred to as 'dynamic response' and the former, 'static'.

According to current ideas the links between the mechanical stimulus and the resulting spindle action potentials are as follows: extension applied to muscle - extension applied to whole spindle - stretching of those parts of the intrafusal fibres on which the sensory endings lie - deformation of sensory terminals - depolarization of sensory terminals - initiation of action potentials, possibly after the electrotonic spread of the receptor potential from its site of origin to the site of initiation of impulses (location unknown). For a sensory ending to have no dynamic response each link in this chain must transmit the stimulus waveform unchanged to the next element. For an ending to have a dynamic response as well as a static response, one of the links must differentiate (in the mathematical sense) the stimulus waveform and add this to the undistorted waveform.

A mechanical origin for the dynamic response of primary endings seems most likely and may be explained by the mechanical properties

of the fibres. The structures of the intrafusal fibres is not the same all along their length, so that their mechanical properties would not be expected to be constant longitudinally. In particular the nuclear-bag region of the nuclear-bag fibres contains far fewer myofibrils than the rest of the fibre. As several authors have pointed out, it might therefore be expected to be less viscous than the polar-striated regions of these intrafusal fibres. If so, the dynamic response of the primary ending is almost a necessary consequence; but the mechanical properties have not yet been examined experimentally.

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