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BLOCKADE BY AMINO ACID ANTAGONISTS OF NEURONAL EXCITATION MEDIATED BY THE PYRAMIDAL TRACT

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SUMMARY

1. The responses to glutamate and amino acid antagonists of cells in the cuneate nucleus of anaesthetized rats have been examined.

2. 1-hydroxy-3-amino-pyrrolidone-2 (HA-966) and glutamic acid diethylester applied by micro-iontophoresis reduced glutamate excitation of the neurones. HA-966 was effective on more cells than glutamic acid diethylester and was more potent. HA-966 did not affect excitatory responses to acetylcholine.

3. Spike activity of cuneate cells was evoked by stimulating the cerebral cortex. Spikes which could be attributed to monosynaptic activation of the cells were studied. The pyramidal tract is the only corticofugal pathway known to be capable of short latency activation of dorsal column nucleus neurones.

4. HA-966 reversibly blocked the evoked activity in twenty-eight (70%) of forty units in which monosynaptically evoked spikes were induced.

5. The results raise the possibility that the neurotransmitter released by neurones of the pyramidal tract may be an excitatory amino acid.

INTRODUCTION

Little attention has been paid to the possible neurotransmitter released by neurones forming the pyramidal tract. The transmitter is not likely to be acetylcholine or a monoamine since neurochemical and histochemical examinations have yielded little evidence for the existence of these substances, or their related enzyme systems, in the tract (Feldberg & Vogt, 1948; Koelle, 1954; Carlsson, Falck & Hillarp, 1962; Dahlström & Fuxe, 1964).

Among the remaining possibilities are the amino acids, and the advent

of amino acid antagonists prompted a previous study of neurones in the cerebral cortex activated by the pyramidal tract (Stone, 1973). In that study it was found that neurones excited by the pyramidal tract appeared to be particularly sensitive to L-glutamate applied by micro-iontophoresis. It was also shown that the synaptic excitation of neurones by pyramidal tract stimulation could be blocked by the amino acid antagonist glutamic acid diethylester (GDEE). However, pyramidal tract interneurones were encountered only rarely in the cortex and, of twenty cells tested, synaptic excitation could be blocked in only five.

The present report is an extension of that study in which the excitation of cells in the cuneate nucleus was examined. Pyramidally excited neurones are more frequently encountered here than in the cortex. Further, the amino acid antagonist 1-hydroxy-3-amino-pyrrolidone-3 (HA-966) has been used as well as glutamic acid diethylester. The former has proved to be much more potent and specific an antagonist than glutamic acid diethylester, and has proved correspondingly more effective in blocking synaptic excitation from the pyramidal tract.

METHODS

Twenty-two male rats weighing 250–300 g were used, and were anaesthetized with urethane, 1·25 g/kg, I.P. Standard surgical techniques were used to expose the left cerebral cortex and the right cuneate nucleus. The animal's rectal temperature was maintained at 37–38° C by means of a heating pad beneath the body. The head was clamped in a stereotaxic frame.

It was found that respiratory and cardiovascular movements of the cuneate nucleus made the holding of units very difficult. All exposed surfaces were therefore covered with a 5 % solution of agar in saline. When gelled the agar was covered with a layer of soft petroleum jelly to prevent the drying out which otherwise occurred, causing distortion of the underlying tissue. With this arrangement it was rarely necessary to use a pressor foot for stabilization.

Details of the micro-iontophoresis technique have been described previously (Stone, 1972*a*, 1973). The five-barrelled micropipettes were filled with the following solutions: sodium L-glutamate (B.D.H.) 200 mM, pH 8·0; acetylcholine chloride (Koch-Light) 200 mM, pH 5·0; L-glutamic acid diethylester hydrochloride (Sigma) 200 mM, pH 5·0; atropine sulphate (Paterson) 100 mM pH 5·0; 1-hydroxy-3-amino-pyrrolidone-2 (Dr J. C. Watkins) 100 mM, pH 4·0 in 0·2 N-HCl.

Glutamate was ejected as an anion (inward current) and other drugs as cations. One barrel was always filled with 200 mM-NaCl at pH 4·0. Current was continuously passed through this barrel such that the net current flow at the five-barrelled tip was always zero. This technique of current balancing (Salmoiraghi & Steiner, 1963) reduces the possibility that the iontophoretic current might itself contribute to changes of cell firing rate. Nevertheless tests were made periodically of the effects of current on a cell by passing a large current (usually 100 nA) through the NaCl barrel. Such controls are illustrated in the appropriate figures. Only one cell was obviously susceptible to current flow and this cell has not been included in the results to be presented.

Since the NaCl was used at a pH of 4·0, the passage of outward current through

the barrel also served as a control for the artificially lowered pH of the HA-966 solution.

Drugs not being ejected were subjected to a retaining current of 15 nA.

Pyramidal tract neurones were excited most conveniently by means of single anodal pulses of 0·1 msec duration applied to the sensory or motor cerebral cortices using a silver ball electrode. Stimulation was achieved from a Devices Digitimer Unit and stimulus isolators.

Three criteria were used to identify cells in the cuneate nucleus which were monosynaptically activated from the cortex. The first was that the minimum latency of the unit should not exceed 5·0 msec. It has previously been shown that the majority of pyramidal tract cells in the rat have antidromic conduction latencies from the medullary pyramid of 0·5–5·0 msec (Stone, 1972*b*). When activated orthodromically, and allowing for conduction from medulla to cuneate plus one synapse, monosynaptically activated cells should have latencies of 1·0 to about 5·5 msec.

The second criterion was that the minimum latency should not vary by more than ± 0·2 msec. This should exclude polysynaptically activated cells from study.

The third criterion was that the recorded spike should follow cortical stimulation at frequencies of 25–100 Hz, but not more than 100 Hz. This test was intended to exclude polysynaptically excited cells and any cells excited antidromically from the cortex.

These tests also served to indicate that the cortical activation of cuneate cells was being achieved via the pyramidal tract, since there is no corticofugal pathway known to be capable of directly exciting dorsal column nucleus neurones other than the pyramidal tract (Jabbur & Towe, 1961; Levitt, Carreras, Liu & Chambers, 1964; Harris, Jabbur, Morse & Towe, 1965; Wiesendanger, 1969).

Extracellular activity was recorded by a single micro-electrode of tip diameter 1 μm fixed alongside the multibarrel electrode (Stone, 1973). Two important advantages of this system are that smaller spikes can be detected than with a conventional centre barrel recording (T. W. Stone, unpublished), and that all five barrels of the multibarrel are available for iontophoresis.

Unit activity was amplified by a Fenlow AD 55 pre-amplifier and the spikes were passed through a pulse shaping and counting unit (Ecko Ratemeter Type N 600B). A continuous recording of cell firing rate was obtained on a Servoscribe pen recorder. Spikes were monitored continuously on Telequipment oscilloscopes and photographic records were taken of cell excitation by the pyramidal tract.

The amplifier bandwidth in all experiments was such that frequencies below 100 Hz were attenuated by approximately 90 %; attenuation at 1 KHz was approximately 50 %.

RESULTS

Amino acid antagonism

The effectiveness of glutamic acid diethylester and 1-hydroxy-3-amino-pyrrolidone-2 (HA-966) as antagonists of excitant amino acids was examined using glutamate as the agonist. Glutamate was applied in pulses of about 5 sec duration every 30 sec with a current of 60 nA. When several consistent responses had been obtained, an antagonist was applied.

Both glutamic acid diethylester and HA-966 were able to antagonize glutamate excitation of cells in the cuneate nucleus, and both substances

exhibited a similar time course of action. Antagonism was apparent after 30–60 sec ejection, and responses to glutamate began to recover 30–60 sec after ending the antagonist ejection, though recovery was often not complete for a further 60 sec.

HA-966 produced a clear antagonism of glutamate on many cells on which glutamate was unaffected by glutamic acid diethylester. Of forty-eight cells tested with the former, antagonism was seen on thirty-five (73 %), whereas glutamic acid diethylester was effective on only fifteen of thirty-eight cells tested (38 %). Records from a cell on which glutamate was blocked by HA-966 but not by glutamic acid diethylester are shown in Fig. 1*A*.

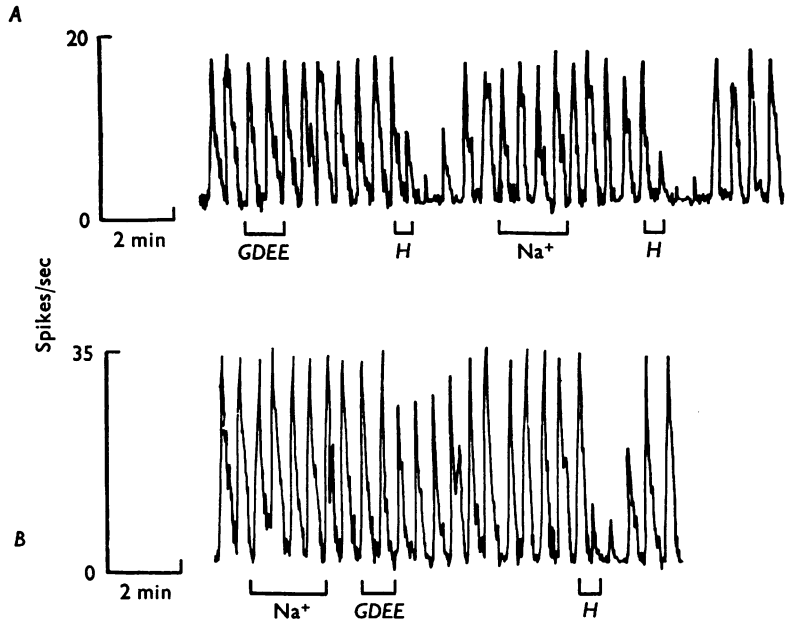

Fig. 1. Records of the firing rate of neurones in the cuneate nucleus which were excited by 5 sec pulses of glutamate, 60 nA. *A*, the glutamate excitation was reduced by the iontophoresis of HA-966 (H), 40 nA, but not by glutamic acid diethyl ester (*GDEE*), 60 nA. A control outward current of 100 nA (Na^+) had no effect on the cell. *B*, glutamic acid diethyl ester, 60 nA only partially antagonized the excitations due to glutamate whereas HA-966, 40 nA, caused an almost complete abolition of the responses. Na^+ was a current control of 100 nA of sodium ions. Time: 2 min.

Even on cells where glutamate responses could be antagonized by both glutamic acid diethylester and HA-966, the latter proved to be more effective as illustrated in Fig. 1*B*. It is not clear whether this difference was due to different antagonist potencies or to different transport numbers

for the two drugs, but, in view of its greater efficacy HA-966 was used for all the subsequent experiments described here.

An ejecting current of 40 nA has been used throughout these experiments since this dose regularly caused an abolition of glutamate responses. Lower doses of, for example, 20 nA often produced only a partial antagonism.

It is very difficult to confirm the specificity of glutamate antagonism by HA-966 in the cuneate nucleus since no substances are known to produce a consistent excitation of cuneate units except the amino acids and

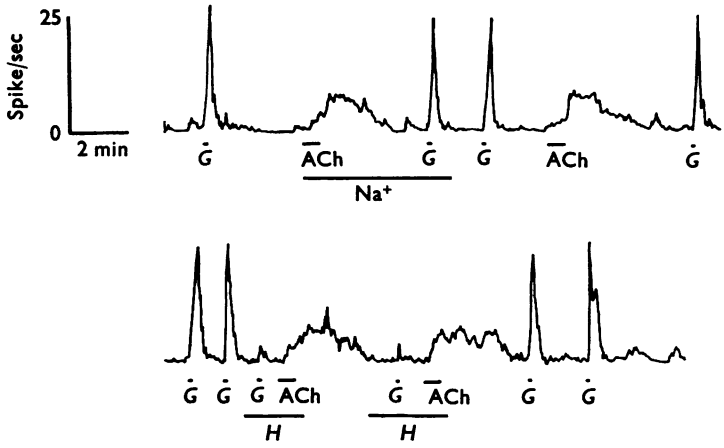

Fig. 2. Records of the firing rate of a cuneate neurone excited by glutamate, 60 nA (*G*) and acetylcholine (ACh) 60 nA. An outward current control of 100 nA (Na^+) had no effect on the cell's responses. HA-966 (H), 40 nA abolished the glutamate excitations without detectably affecting the acetylcholine responses. Time: 2 min.

some chelating agents (Galindo, Krnjević & Schwartz, 1966). However, as found by previous groups (Galindo *et al.* 1966; Steiner & Meyer, 1966) a small number of cells (six) was encountered on which acetylcholine produced an increase of firing rate, albeit much weaker than seen in, for example, the cerebral cortex (Krnjević & Phillis, 1963; Crawford & Curtis, 1966; Stone, 1972*a*).

On all these six cells, HA-966 could be ejected with a current of 40–50 nA to produce a 60–90 % reduction of glutamate responses with no detectable effect on the acetylcholine responses. Records from one of these units are shown in Fig. 2. On two of the acetylcholine sensitive cells atropine was applied by iontophoresis with a current of 40 nA and, in both cases, the acetylcholine responses were reduced with only a small (about 10 %) reduction of the glutamate responses.

The neuronal responses to glutamate seen in the present experiments were somewhat different from responses seen by previous groups (e.g. Curtis, Johnston, Game & McCulloch, 1973) and perhaps deserve comment. The onset of responses was usually rapid. This is reflected in the rapid rise time of the response records, to which switching artifacts contributed negligibly. The relatively slow offset of the responses was partly due to a time constant of decay in the counting circuit of approximately 4 sec, and partly to a truly slow decay of the amino acid responses. This latter in turn may be partly a neuronal phenomenon and partly a physical one due to diffusion of the drug after ending ejection. The over-all responses are similar to those seen previously in the cerebral cortex (Stone, 1973).

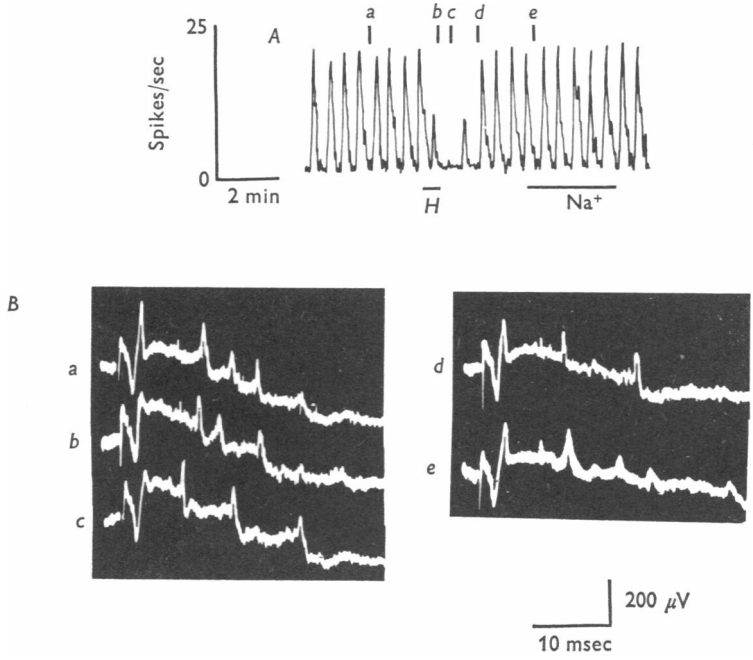

Fig. 3. *A*, record of the firing rate of a neurone excited by pulses of glutamate, 60 nA. The responses were greatly reduced by HA-966 (H), 40 NA, but unaffected by an outward current control of 100 nA(Na^+). Time, 2 min.

B, photographs of the responses of the neurone to activation of corticofugal fibres, taken at the time indicated in *A*. In *a* is seen an early monosynaptic spike followed by polysynaptically evoked spikes. In *b* and *c* taken when glutamate excitation was blocked by HA-966, the early monosynaptic spike has also been blocked, but the later spikes show that HA-966 was not affecting spike height at this time. The early spike reappeared as the glutamate responses reappeared in *d*. Outward current did not affect the cell (*e*). The spikes in *d* and *e* are slightly smaller than in *a*–*c* due to slight movement of the electrode. Calibrations: 200 μV, negativity upwards; and 10 msec.

Synaptically evoked activity

Following single anodal shocks delivered to the surface of the cerebral cortex, short latency spikes were evoked in cells in the deeper parts of the cuneate nucleus. Spikes which satisfied the criteria for monosynaptic excitation were evoked in forty of 112 cells encountered, and all but three of these were found at depths of 400–800 μm. In a further twenty-two units spike activity could be evoked which was clearly not monosynaptic and, in the remaining fifty cells, no spike activity could be consistently related to the stimulus using stimulus strengths up to the level at which movement of the animal was produced (about 200 μA).

Fig. 3 illustrates the induced activity and glutamate sensitivity of a cell found at a depth in the cuneate nucleus of 660 μm from a surface position 1·9 mm caudal to the obex and 1·5 mm from the mid line. The first evoked spike was monosynaptically induced. Subsequent spikes were presumably due to activation over polysynaptic pathways, since the latencies varied by several msec with successive stimuli, and none would follow stimuli at frequencies of more than 10 Hz. Of the forty units in which monosynaptic activity was evoked, eleven also showed such later polysynaptic activity. It can be seen from Fig. 3 that the iontophoresis of HA-966 reduced glutamate excitation after the usual 30–60 sec, and that the monosynaptically induced spike also disappeared at this time. The later spikes could still be evoked however, indicating that HA-966 was not having any direct depressant action on the cell's firing or spike height. Since the later spikes were also synaptically induced, it also follows that HA-966 was showing a degree of specificity for the direct corticocuneate synapses, and was not blocking indiscriminately all synaptically evoked activity.

The activity of a second cell is illustrated in Fig. 4. The iontophoresis of HA-966 to this cell produced a particularly rapid blockade of glutamate excitation, and also a rapid blockade of the synaptically induced activity. The evoked spike disappeared quite suddenly about 15 sec after starting the ejection of HA-966. Again there was no change of spontaneous firing rate or of spike height at this time. Both the spike activity and the glutamate responses returned about 30 sec after ending the antagonist application (Fig. 4). Fig. 4 also shows that the iontophoresis of atropine, 40 nA for 1 min, had no effect on the induced spike activity.

Of the forty units in which suitable spike activity was induced, HA-966 blocked that synaptic activation on twenty-eight (70%). Glutamate excitation was reduced by at least 50% on all these cells at the time of synaptic blockade. On the remaining twelve units on which synaptic activity was not blocked glutamate responses were reduced in nine and almost unaffected by HA-966 in three cells.

Atropine was applied to six units and in none of these cells was there any apparent change of the evoked activity (Fig. 4).

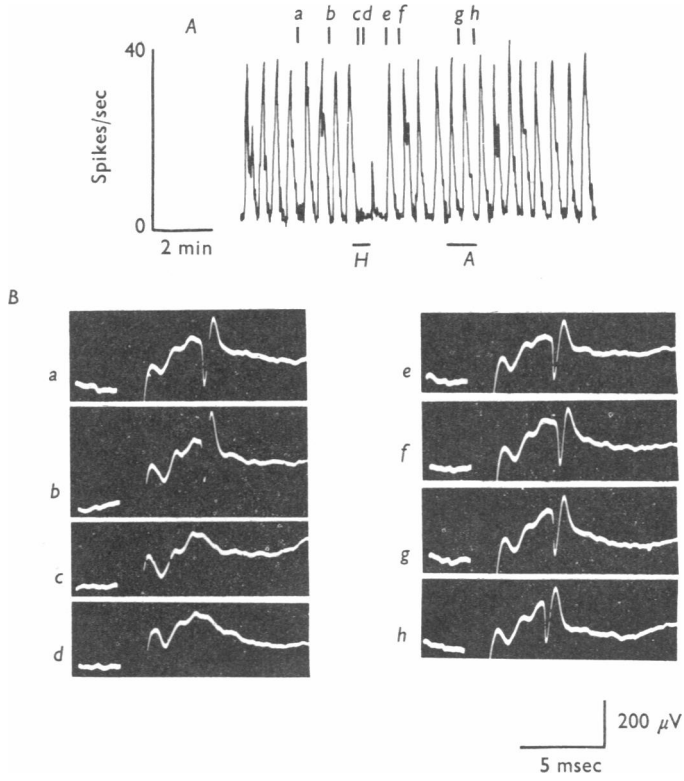

Fig. 4. *A*, record of the firing rate of a neurone excited by pulses of glutamate, 60 nA. Responses were greatly reduced by HA-966 (*H*), 40 nA, but unaffected by atropine (*A*), 40 nA. Time, 2 min.

B, photographs of the responses of the neurone to activation of corticofugal fibres taken at the times indicated in the upper trace. In *a* and *b* are seen an early monosynaptic spike. In *c* and *d* this spike was blocked at the same time as blockade of the glutamate responses in the upper trace. Records *e* and *f* show the return of the spike as the glutamate responses returned and *g* shows that the spike was not blocked by atropine, 40 nA. Calibration: 200 μV, negativity upwards; and 5 msec.

DISCUSSION

Amino acid antagonism

HA-966 is probably the most satisfactory antagonist of excitatory amino acids available at present. It has previously been shown to antagonize amino acid excitation of neurones in the cat cerebral cortex (Davies &

Watkins, 1972, 1973*a*), cuneate nucleus (Davies & Watkins, 1973*a*, *b*) and spinal cord (Curtis *et al.* 1973). These workers have found that HA-966 can be effective as an amino acid antagonist at doses which do not have direct effects on the neuronal membrane to cause changes of spike height, and which have little effect on the resting firing rates of cells. The present results have confirmed these observations in the cuneate nucleus of rats.

Although a demonstration of the specificity of HA-966 was restricted by the relative insensitivity of cuneate neurones to acetylcholine (Galindo *et al.* 1966; Steiner & Meyer, 1966) six cells were encountered which were sensitive to acetylcholine and on all these cells acetylcholine responses were unaffected by doses of HA-966 adequate to reduce glutamate responses. HA-966 has previously been shown to exhibit selectivity as an antagonist of amino acid excitation compared with acetylcholine excitation in the cat cerebral cortex (Davies & Watkins, 1972, 1973*a*). Some specificity has been observed on Renshaw cells in the cat spinal cord, but this specificity seems to be less marked than in the cortex or cuneate nucleus (Curtis *et al.* 1973).

In the present experiments HA-966 was able to antagonize glutamate excitation on a larger proportion of cells than was glutamic acid diethylester, and the potency of HA-966 on individual cells was greater. Similar observations were reported by Davies & Watkins (1973*a*). This apparent potency difference may be a true difference of antagonist potency at amino acid receptors, or it may reflect a higher transport number for HA-966. Whatever the explanation, the relatively poor performance of glutamic acid diethylester as an antagonist may explain the failure of this substance to block more than a small proportion of cells in the cerebral cortex synaptically driven from the pyramidal tract (Stone, 1973).

The mechanism by which HA-966 reduces amino acid excitation has been discussed by Davies & Watkins (1973*a*). Interaction is likely to be a true pharmacological antagonism since γ-aminobutyric acid and glycine, which may be considered as physiological antagonists, show no detectable selectivity for amino acids or acetylcholine. Further evidence that HA-966 is not likely to be acting on inhibitory amino acid receptors is shown by the failure of strychnine and bicuculline to prevent the antagonism of glutamate by HA-966 (Davies & Watkins, 1973*a*).

Synaptically evoked activity

The possibility must be considered that the stimulation of the cerebral cortical surface was inducing activity in the cuneate nucleus over pathways other than the pyramidal tract. This is unlikely since Jabbur & Towe (1961) and Levitt *et al.* (1964) have shown that cortically induced excitation of cells in the dorsal column nuclei was abolished by sectioning the

pyramidal tract. The pyramidal tract is known to send collaterals to the dorsal column nuclei and to be capable of monosynaptically exciting neurones in those nuclei (Harris *et al.* 1965).

That cells were being activated by the pyramidal tract is supported by the fact that all the short latency spikes recorded in the present experiments were from cells lying relatively deeply in the cuneate nucleus. Walberg (1957) and Kuypers (1958) have shown that pyramidal tract axons terminate in these deeper layers of the dorsal column nuclei.

The antagonism by HA-966 of synaptically induced neuronal activity therefore suggests that glutamate or a related excitatory amino acid might be the neurotransmitter released by fibres of the pyramidal tract. It is not possible to specify a single amino acid since none of the antagonists so far examined, including HA-966, can distinguish between glutamate, aspartate and DL- homocysteate excitation (Davies & Watkins, 1972, 1973*b*; Curtis *et al.* 1973).

It is interesting to note that HA-966 did not interfere with the activation of cuneate neurones via polysynaptic pathways (Fig. 3). Since cholinergic and amine-containing fibres do not seem to be prominent among axons entering the cuneate nucleus (Feldberg & Vogt, 1948; Koelle, 1954; Carlsson *et al.* 1962; Dahlström & Fuxe, 1964), the possibility is raised that a novel transmitter substance may be elaborated by the interneurones involved.

Glutamate is certainly known to occur in the dorsal column nuclei (Johnson & Aprison, 1970), although this fact has usually been considered to support evidence that glutamate might be the transmitter released by primary afferent fibres (Duggan & Johnston, 1970; Roberts, Keen & Mitchell, 1973; Davies & Watkins, 1973*a*, *b*).

Primary afferent fibres do not ascend to the cerebral cortex however, and yet glutamate is present in the cortex in synaptosomes (Bradford & Thomas, 1969). Jasper & Koyama (1969) have shown that glutamate can be collected at the surface of the cortex and that stimulation of the reticular formation but not the mesial thalamus increases the rate of 'release'. Also some cortical neurones are extremely sensitive to the iontophoresis of glutamate and can be excited for several seconds by a pulse of glutamate lasting only 50 msec (Stone, 1973).

These facts, together with the present results, raise the possibility that an excitatory amino acid may be the pyramidal tract neurotransmitter.

I should like to thank Dr J. C. Watkins for the gift of HA-966.

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