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Problems in the Evaluation of Glutamate as a Central Nervous System Transmitter

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There is now an extensive literature concerned with the excitation of neurones in the mammalian CNS by L-aspartate, L-glutamate, and by straight-chain and cyclic analogs of these naturally occurring acidic amino acids.

When the depolarization of single neurones by microelectrophoretic aspartate and glutamate was first reported (21), a role for these amino acids as excitatory transmitters was considered unlikely, despite the presence of substantial amounts of each within the brain. The major reason was the very close similarity in the time courses of recovery from excitation by D- and L-enantiomorphs of these amino acids (see also ref. 11), which was considered to exclude extracellular enzymic degradation as a means of inactivating synaptically released aspartate or glutamate. It is now clear, however, that extracellular enzymic modification, as occurs at cholinergic synapses, is of little or no significance at central synapses utilizing amino acids as transmitters (18). Under *in vitro* conditions, both low- and high-affinity sodium-dependent transport processes have been described for excitatory and inhibitory amino acids (66), and the similar time courses of recovery from excitation by microelectrophoretic D- and L-aspartate, D- and L-glutamate, and L-homocysteate most likely result from the sharing by these amino acids of a low-affinity, high V_{max} uptake mechanism (2,11).

Another reservation regarding a transmitter function for the excitant amino acids was the large variety of neurones that were excited by aspartate and glutamate, but this may well reflect the widespread occurrence of "aspartergic" and "glutamergic" neurones within the mammalian CNS, rather than the nonphysiological, "nonspecific," nature of the excitation. Since both aspartate and glutamate are possibly excitatory transmitters, it has been convenient to classify the appropriate postsynaptic receptors as "aspartate-" and "glutamate-preferring" (47). Both amino acid molecules are flexible; if that of glutamate interacts with the glutamate receptor in an extended conformation, interaction with the aspartate receptor would be possible in a folded conformation. Aspartate, however, having a shorter chain length, would be unlikely to interact with such a glutamate receptor, and aspartate and N-methyl-D-aspartate are possibly highly selective for aspartate-preferring sites.

On the other hand, kainate, a conformationally restricted analog of glutamate, may be more selective for a glutamate-preferring receptor.

In this brief review I wish to outline the investigations of the mode of action of excitant amino acids that may be relevant to their toxic effects on neurones, the use of agonists and antagonists to study aspartergic and glutamergic pathways *in vivo*, and neurochemical evidence for such pathways in certain regions of the brain.

DEPOLARIZATION OF NEURONES BY AMINO ACIDS

There are major technical difficulties in investigating the ionic mechanism of the synaptic excitation of central neurones *in vivo*, largely because of the somatic and dendritic location of excitatory synapses. The same investigational problems confound attempts to compare the ionic mechanism of amino acid depolarization with that induced synaptically. Although it is probable that not all excitatory transmitters have an identical postsynaptic action (see ref. 52), at those synapses which have been most thoroughly investigated there appears to be an increase in the membrane permeability to sodium and potassium ions.

The reversible depolarization of spinal, cortical, and caudate neurones by aspartate and glutamate is accompanied by an increase in membrane conductance, and the measured "reversal" potential is not inconsistent with an increased membrane permeability to both sodium and potassium ions (4,14,21,53,63,93). The involvement of chloride ions seems unlikely, and, since tetrodotoxin does not block the depolarization, the sodium permeability increase differs from that associated with action potentials (17,92). The participation of sodium ions is also apparent from the observation that the depolarization of cultured human and rat spinal neurones by aspartate and glutamate can be abolished reversibly by replacement of extracellular sodium with choline (41). The enhancement of the excitatory effects of L-aspartate and L-glutamate on spinal neurones by *p*-mercuriphenylsulfonate, an inhibitor of amino acid uptake, suggests that the depolarization is not, however, generated by a carrier-linked transport of sodium and amino acids (18).

Although the failure to excite neurones by a number of agents that are more powerful calcium chelators than glutamate suggests that the excitation is not produced merely by a lowering of the extracellular calcium ion concentration (20), the interaction between excitant amino acid molecules and external membrane receptors may initiate a sodium ion permeability increase by displacing calcium ions from critical membrane sites (22).

Thus, the ionic mechanism of the depolarization of neurones by amino acids *in vivo* may well be identical to that produced by some synaptically released transmitters. With cultured mouse spinal neurones, however, the discrepancy between the reversal potentials for synaptic and glutamate depolarizations suggests different ionic mechanisms and that this amino acid is not a naturally occurring synaptic transmitter (75).

A number of recent findings suggest that the excitatory effect of acidic amino acids is not as simple as originally proposed and that several other factors have to be taken into consideration in addition to the interaction with membrane receptors and

the initiation of a change in membrane permeability. These include ion movements linked with amino acid uptake and the disturbances of intra- and extracellular ion concentrations produced by prolonged activation of receptors and excitation of neurones.

Uptake may be very important in determining the effectiveness of an amino acid as an excitant. The only available technique for comparing the potencies of amino acid excitants *in vivo* is electrophoretic administration into the vicinity of single neurones. Assuming a similarity of transport number (13), the potencies are expressed relative to glutamate by comparing equally effective electrophoretic currents. Potency ratios determined in this fashion, however, are not based on a comparison of equieffective amino acid *concentrations*, since the concentration of a particular amino acid near the membrane receptors depends on both its rate of ejection from the micropipette and the rate of removal from the vicinity of receptors by uptake and other factors.

Thus, the high potencies of N-methyl-D-aspartate, D-homocysteate (23), β-N-oxalyl-L-α,β-diaminopropionate (86), ibotenate (48), kainate (47,81), and quisqualate and domoate (8) may to a considerable extent reflect the absence of appropriate uptake mechanisms, as has been demonstrated *in vitro* for D-homocysteate (8) and kainate (49). With amino acids such as these, cotransport with sodium is unlikely to contribute significantly to the depolarization. A similar consideration may apply to the relative toxicity of amino acids; the parallelism demonstrated between excitant and toxic potencies (70,71,77) suggests that excitation *per se* may be more important for the destruction of cells than intracellular metabolic disturbances produced by an amino acid after uptake.

A recent investigation indicates that the depolarization of spinal motoneurones by D-homocysteate, unlike that induced by L-glutamate, is associated with a *decrease* in membrane conductance (54), and other differences between excitatory amino acids have become apparent in both *in vivo* and *in vitro* studies of the effects of magnesium ions and of changes in external sodium and potassium ion concentrations on amino acid excitation. Using the hemisected frog or immature rat spinal cord *in vitro*, in the presence of either procaine or tetrodotoxin, amino acids that depolarize motoneurones have been classified into three types on the basis of the effects of altering the potassium and sodium ion concentrations in the bathing medium (28). Depolarization by group I amino acids (D- and L-aspartate, L-glutamate, L-cysteate and L-cysteine sulfinate) was enhanced in K^+-free media and reduced in low-Na^+ media; that by group II amino acids (N-methyl-D-aspartate, D-homocysteate, and quisqualate and kainate) was unaltered in K^+-free media and reduced in low-Na^+ media; that by group III amino acids (D-glutamate, L-homocysteate, and L-homocysteine sulfinate) was either unaffected or increased in K^+-free media and *enhanced* in low-Na^+ media. Such results suggest the importance of both an induced permeability increase to sodium ions (group I and II) and ion-dependent uptake (unlikely with group II) to the depolarizing effect of amino acids, but the significance of these *in vitro* studies to the *in vivo* situation requires further investigation.

Under similar *in vitro* conditions, low concentrations of magnesium (0.5–1.00

mM) depressed the depolarizing effects of N-methyl-D-aspartate, D-glutamate, D-aspartate, and L-homocysteate, but had little or no effect on the actions of L-glutamate, kainate, or quisqualate (29). The action of L-aspartate was reduced less than that of N-methyl-D-aspartate, and to a significantly greater degree than that of L-glutamate. Since this effect of magnesium does not correlate with known characteristics of amino acid uptake, and, being independent of calcium concentration, is unlikely to result from a presynaptic transmitter-releasing effect of excitant amino acids, Evans et al. (29) suggested the presence of magnesium-sensitive and magnesium-insensitive postsynaptic receptors for excitant amino acids. A subsequent study using cat spinal neurones *in vivo* established similar differences in sensitivity to the elevation of extracellular magnesium ion concentrations between the excitant effects of N-methyl-D-aspartate (reduced by magnesium) and kainate (unaffected), and the action of L-aspartate was reduced to a greater extent than that of L-glutamate (25).

Although these observations are generally consistent with the presence of at least two major types of excitant amino acid receptors (aspartate-preferring, magnesium-sensitive; glutamate-preferring, magnesium-insensitive), the finding that high magnesium concentrations also reduced the sensitivity of Renshaw cells to acetylcholine (25) suggests that the interpretation of the effects of magnesium ions in terms of an influence at excitant amino acid receptors may be an oversimplification.

DEPOLARIZATION OF AFFERENT TERMINALS BY AMINO ACIDS

The recent demonstration that aspartate, glutamate, and other acidic amino acids depolarize primary afferent terminals in the ventral and dorsal horns of the cat spinal cord (19) is also relevant to the possible transmitter functions of these amino acids. Afferent terminals are depolarized by GABA, and this effect, blocked by bicuculline, is consistent with the participation of GABA as a depolarizing transmitter at axo-axonic synapses concerned with "presynaptic" inhibition (see ref. 16). GABA also depolarizes dorsal root ganglion cells at bicuculline-sensitive nonsynaptic sites. In contrast, although dorsal root ganglion cells are insensitive to acidic amino acids both *in vivo* (P. Feltz, *personal communication*) and in culture (56,75), primary afferent terminals *are* depolarized. The effect is not blocked by bicuculline, and, contrary to the preliminary report (19), the relative depolarizing potencies of different acidic amino acids appear to be similar to that of their actions as neuronal excitants. In particular, kainic acid has a very powerful and reversible depolarizing action (Curtis and Lodge, *unpublished*), yet apparently is not neurotoxic to synaptic terminals (31,72). Depolarization of terminals by these excitants appears not to involve an increase in membrane conductance, as is the case with depolarization of GABA.

Further investigation is required to determine the significance of this depolarization, and particularly whether it releases transmitter, or enhances or decreases transmitter release by presynaptic impulses. It will be necessary to ascertain whether

other excitatory axo-axonic synapses occur in addition to those at which GABA is a transmitter. The depolarization may be nonsynaptic, but nevertheless an indication that the depolarized terminals *are* aspartergic or glutamergic. Such an action may be entirely nonphysiological. On the other hand, however, it may be important in the control of transmitter release (42,55). In the case of Renshaw cells, a release of acetylcholine from axon collateral terminals by excitant amino acids seems most unlikely, since the excitation of these cells by amino acids is not reduced significantly by dihydro-β-erythroidine, an acetylcholine antagonist.

EXCITANT AMINO ACID ANTAGONISTS

Specific antagonists effective at aspartate- and glutamate-preferring receptors are required not only for studying the actions of excitant amino acids, but also for distinguishing aspartergic from glutamergic synaptic excitation.

The initial studies in this field were concerned predominantly with distinguishing amino acid-induced excitation from that produced by other substances, especially by acetylcholine, the effects of which could be selectively blocked by either dihydro-β-erythroidine or atropine (15). Several compounds have been proposed as relatively specific excitant amino acid antagonists, but consistent results have rarely been obtained by different investigators, and until comparatively recently, none has been generally accepted as suitable for providing unequivocal evidence that a particular synaptic excitation involved one or the other excitant amino acid as the transmitter.

This type of investigation has been limited by technical problems (15). Many agents having depressant effects on the CNS, possibly arising from a selective action at excitatory synapses, are of low aqueous solubility, and hence are unsuitable for testing microelectrophoretically. More importantly, using microelectrophoretic techniques, it is difficult to investigate membrane receptors located on the dendrites of neurones, and many possible antagonists have been studied under conditions where a relatively high perisomatic concentration may have obscured specific effects at dendritic synapses, particularly those of morphologically complex neurones such as motoneurones and pyramidal and Purkinje cells. There has also been a tendency to extrapolate results from one kind of neurone in a particular species, even of invertebrates, to all neurones in the mammalian CNS.

Compounds proposed as antagonists include (+)-lysergic acid diethylamide, α-methyl-DL-glutamate, L-glutamate diethyl ester, L-methionine-DL-sulfoximine, 9-methyoxyaporphine, apomorphine, 5,6-dimethoxyaporphine (nuciferine), and 1-hydroxy-3-aminopyrrolid-2-one (15,63). More recently, other substances tested include SP-III, a water-soluble derivative of delta-tetrahydrocannibinol (80), 2-amino-4-phosphonobutyrate (87), D-α-aminoadipate (5,35), and α,ϵ-diaminopimelate (5). Unfortunately, until recently, few investigators have made use of amino acid *agonists* having relatively specific effects at aspartate- and glutamate-preferring receptors when testing these antagonists.

Although initial experiments indicated that 5,6-dimethoxyaporphine was of little

use in distinguishing amino acid from cholinergic excitation of Renshaw cells in the rat (27) and cat (D. R. Curtis, *unpublished*), some selectivity towards amino acid excitation has been observed in the thalamus (3,65), cuneate nucleus (40), optic tectum of the pigeon (30), and spinal cord (74).

Considerable use has been made of L-glutamate diethyl ester as an excitant amino acid antagonist (17,33,34,62,63,65,78,82,83). Although the usefulness of this substance has been questioned in that there is relatively little difference between concentrations selectively blocking amino acid effects and those having a nonselective action (see refs. 1,10,17,65), this small difference may be more a reflection of the problems of the administration of antagonists microelectrophoretically (15) than of the small degree of selectivity of this glutamate analog. In a more recent study in the rat thalamus (39), L-glutamate diethyl ester has been shown to be less effective as an antagonist of N-methyl-D-aspartate (and kainate) than of L-glutamate.

In contrast to these results, D-α-aminoadipate appears to be a more effective antagonist of the excitatory action of N-methyl-D-aspartate than of L-glutamate and kainate (5,6,7,35,39,57). The diamino acid, α,ϵ-diaminopimelate has a similar but slightly different spectrum of antagonism (5,6). Although there is some controversy regarding the effect of D-α-aminoadipate on the sensitivity of Renshaw cells to acetylcholine (6,57), this amino acid and analogs of relatively simple structure may prove of considerable value in assessing the relative importance of aspartate and glutamate receptors on particular kinds of neurone.

PHARMACOLOGICAL INVESTIGATION OF AMINO ACID PATHWAYS

Although virtually all neurones so far tested *in vivo* are excited by L-aspartate and L-glutamate, certain differences in the relative sensitivities of some neurones to these excitants suggest a predominance of one type of amino acid-releasing termination on them. It is unfortunate, however, that in few of these studies has use been made of agonists that may have selective effects on either aspartate- or glutamate-preferring receptors. This type of investigation, together with the use of antagonists, has provided some information regarding amino acid excitatory pathways.

In the cat ventrolateral thalamus, neurones excited synaptically from the brachium conjunctivum were more sensitive to L-glutamate (and acetylcholine), relative to DL-homocysteate and N-methyl-DL-aspartate, than cells located dorsally, thus suggesting that the excitatory fibers of the brachium may be glutamergic (35,64). This proposal gained support from the reduction of both the sensitivity of these neurones to L-glutamate and their firing by impulses in the brachium conjunctivum by L-glutamate diethyl ester (35).

Antagonism by L-glutamate diethyl ester has also been used to support claims for the involvement of excitant amino acids as transmitters of a number of other pathways: the commissural pathway to the rat hippocampus (78), a corticostriatal pathway in the rat (82), and the perforant pathway to the rat dentate gyrus (67). Additionally, reduction by l-hydroxy-3-aminopyrrolid-2-one of the excitation of

cuneate neurones by pyramidal tract volleys (83), and by SP-III of responses generated in the rat hippocampus by commissural volleys (80), have also been used to support the involvement of amino acid excitatory transmitters.

In the spinal cord of the cat, Renshaw cells, which probably are not excited monosynaptically by impulses in dorsal root primary afferent fibers but only polysynaptically after excitation of excitatory interneurones, were less sensitive to L-glutamate than to L-aspartate, dorsal horn interneurones having the opposite order of sensitivity (26). This observation, and the subsequent finding that dorsal horn interneurones were more sensitive than Renshaw cells to kainate, relative to N-methyl-D-aspartate (59), is consistent with the presence of glutamergic primary afferents and aspartergic excitatory spinal interneurones. This conclusion is also supported by recent findings that D-α-aminoadipate selectively reduced the long-latency synaptic firing of Renshaw cells by dorsal root volleys, without altering the cholinergic excitation produced by ventral root stimulation (6,7,57). In the rat, however, although dorsal horn interneurones have been reported to be more sensitive to L-glutamate than to L-aspartate, and Renshaw cells to be equally sensitive to these amino acids (9), the results of another study indicate that the two kinds of neurone cannot be distinguished on the basis of sensitivity to amino acids (44).

NEUROCHEMICAL EVIDENCE FOR ASPARTERGIC AND GLUTAMERGIC PATHWAYS

Although it is difficult to distinguish transmitter from metabolic pools of aspartate and glutamate within neurones, if indeed there are separate pools of these types for each amino acid, the association of these amino acids with particular neurones may be revealed by the changes in distribution that follow specific nucleus or pathway lesions. Furthermore, after such lesions, there may be alterations in the uptake of transmitter amino acids *in vitro* as a consequence of the loss of excitatory terminals, and a reduction of the *in vitro* depolarization-induced release of amino acids, a process that while not necessarily directly related to synaptic release *in vivo*, may nevertheless provide a measure of the presynaptic store of amino acid transmitters.

In the spinal cord of the cat, the high levels of glutamate in dorsal roots and the dorsal horn relative to those ventrally, the higher levels of aspartate in the ventral horn than dorsally, and the correlation between reduced aspartate levels and the loss of central interneurones produced by temporary cord hypoxia suggested that glutamate may be the transmitter of some primary afferents, and aspartate that of some excitatory spinal interneurones (24,45,46). Although dorsal root section had little effect on dorsal horn glutamate levels, glutamergic primary afferent terminals may contain a very small fraction of the total glutamate within the cord (46), and the proposal regarding the functions of these two amino acids are consistent with the neuropharmacological investigations mentioned above.

After unilateral ablation of the cochlear, and degeneration of primary synapses within the ventral cochlear nucleus of the guinea pig, aspartate and glutamate levels

in this region were significantly reduced, those of aspartate paralleling the morphological changes (88,89). Some cochlear afferents within the auditory nerve may thus be aspartergic (see also ref. 32).

When neonatal hamsters were infected intracerebrally with a rat parvovirus, there was a more than 90% loss of cerebellar granule cells, together with a profound fall in cerebellar glutamate levels by as much as 40% and the uptake of aspartate and glutamate by cerebellar homogenates (91). Cerebellar levels of glutamate in the rat were significantly reduced by prior X-irradiation, which selectively reduced the number of granule cells (85). Cerebellar glutamate levels were also low in mutant mice having a reduced number of granule cells (43,76), and all of these observations suggest that the granule cell synapses with Purkinje cell dendrites may be glutamergic. In contrast, cerebellar cortical aspartate levels were low in rats after destruction of the inferior olive with 3-acetylpyridine (68), and in humans with inherited olivopontocerebellar atrophy (73), suggesting that climbing fibers, which also synapse on Purkinje cell dendrites, are aspartergic.

In the rat, lesions of corticostriatal pathways produced a significant reduction in the uptake of glutamate by tissue from the caudate-putamen (61), and frontal cortical ablation resulted in a decrease in striatal glutamate levels (50), suggesting the glutamergic nature of a corticostriatal pathway. There appears to be a requirement for this tract to be intact for intrastriatal kainate to destroy neurones (60), although, in the cerebellum, kainic acid apparently destroyed all cells except granule cells, which may be glutamergic (38). The former observation suggests that glutamergic *terminals* need to be present in order for kainate to be neurotoxic, possibly via the release of glutamate, whereas the latter suggests a requirement for postsynaptic glutamate *receptors*. Granule cells, however, are excited by DL-homocysteate (12) and L-glutamate (58), and thus presumably bear glutamate receptors.

In the hippocampal formation, the distribution of glutamate; of zinc, which may have a role in relation to moss fiber terminals; and of labeled glutamate in both normal tissue and after lesions of afferent pathways suggested that the perforant pathway, the granule cells, and the pyramidal cells of areas CA3 and 4 may be aspartergic or glutamergic (84). Studies *in vitro* of the uptake and efflux of aspartate and glutamate by hippocampal slices, and of the effects of chronic lesions of commissural fibers and the perforant path, indicate the possibility that the former (from pyramidal cells) is aspartergic; the latter, glutamergic (36,69). Although the excitation of granule cells in hippocampal slices by stimulation of the perforant path was reduced by DL-2-amino-4-phosphonobutyrate (90), this phosphonic analog of glutamate could not be demonstrated to be a glutamate antagonist in either the cat spinal cord (87) or the rat hippocampus *in vivo* (79).

The substantial reduction in the levels of aspartate and glutamate in the guinea pig olfactory cortex following removal of the olfactory bulb has suggested the presence of aspartergic and glutamergic fibers in the lateral olfactory tract (37). Similarly, changes in the glutamate content of the cerebral cortex after undercutting may be associated with the destruction of afferent glutamergic pathways (51).

CONCLUSION

Although the evidence for transmitter roles of aspartate and glutamate is far from convincing, there is sufficient information to make such functions seem probable. Aspartergic and glutamergic fibers may constitute the main central afferent and efferent pathways within the mammalian CNS, pathways that appear not to involve any other accepted transmitter, such as acetylcholine or the biogenic "amines."

In view of the primary function of the brain as an information processing system, it would not be unexpected that substances hitherto considered mainly of metabolic significance could be important in the actual transfer of "data" at synapses. The use of commonly available and relatively simple molecules would provide reliability in a system in which effectiveness and subtlety depended on the morphology and location of synapses, rather than on the nature of the transmitters. Furthermore, uptake may provide an efficient method for transmitter inactivation, and recycling, without the necessary participation of catabolic and anabolic enzymes.

Blood-brain barriers isolate central synapses from circulating synaptically important amino acids, and extraneuronal concentrations are maintained at relatively low levels by cellular uptake mechanisms that are specific for particular classes of amino acid. Defects in either these barriers or uptake processes, together with abnormally high systemic or local concentrations of natural or unnatural excitant amino acids, could result in excessive discharge of neurones, and the consequent disturbances of the normally well-regulated intracellular ion and metabolite levels may result in cell death.

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