Br. J. Pharmac. (1980), **68**, 711–717

STRUCTURE-ACTIVITY STUDIES ON AN EXCITATORY RECEPTOR FOR GLUTAMATE ON LEECH RETZIUS NEURONES

VICTORIA A. JAMES, R.J. WALKER & H.V. WHEAL

School of Biochemical and Physiological Sciences, University of Southampton, Southampton, S09 3TU

1 Intracellular recordings were made from Retzius cells from the segmental ganglia of *Hirudo medicinalis* and *Haemopis sanguisuga*. Glutamate had a direct excitatory effect on the leech Retzius cells.

2 L-Glutamate was 25 times more potent than D-glutamate.

3 L-Glutamate was approximately equipotent with ibotenic acid and 11.2 times more potent than L-aspartic acid.

4 Quisqualic acid and kainic acid were both approximately 100 times more potent than L-glutamate. DL-1-Amino-*cis*-1,3-dicarboxycyclopentane was approximately 5 times more potent than L-glutamate, while the *trans* isomer was 105 times less potent.

5 α-NH_2-pimelic acid and β-CH_3-glutamic acid reduced the response to L-glutamate.

6 It is suggested that glutamic acid may interact with the Retzius cell glutamate receptor in an extended conformation.

Introduction

Glutamate receptors are present in a wide range of animal tissues (Gerschenfeld, 1973; Curtis & Johnston, 1974; Usherwood, 1977; 1978). There is evidence to suggest that there are two types of glutamate receptor, 'aspartate'-preferring receptors and 'glutamate'-preferring receptors, (Morgan, Vrbova & Wolstencroft, 1972; Johnston, Curtis, Davies & McCulloch, 1974). 'Glutamate'-preferring receptors possess a high affinity for glutamate in an extended conformation (McCulloch, Johnston, Game & Curtis, 1974) whilst 'aspartate'-preferring receptors have a high affinity for glutamate in a folded conformation (Johnston *et al.*, 1974).

Conformationally restricted analogues of glutamate help to provide evidence in determining the type of glutamate receptor present in a tissue. Results of a structure-activity study on leech Retzius cells with conformationally restricted analogues suggest that the receptor for glutamate at this site is 'glutamate'-preferring. A preliminary note of some of this work has already been published (James & Walker, 1978).

Methods

All experiments were performed on the isolated segmental ganglia of the leech *Hirudo medicinalis* or *Haemopis sanguisuga*. The leeches were pinned ventral side down on to a wax block and dissected by a dorsal-longitudinal incision. The viscera were cleared aside to expose the ventral nerve cord enclosed in its blood sinus. The blood sinus was then dissected away and short segments of 3 ganglia were attached ventral side uppermost to a glass slide by means of small elastic bands across the connective nerves. Nicholls & Kuffler (1964) have shown that compounds added to the bathing medium easily reach the neurones of the leech ganglia without further dissection. The glass slide with the leech ganglia was then placed in the experimental bath (volume 20 ml) and viewed through an Olympus stereo-zoom binocular microscope. The ganglia were bathed in leech Ringer (mM): NaCl 115, KCl 4, $CaCl_2$ 2, glucose 10, Tris HCl 10, pH 7.4.

The two Retzius cells of each segmental ganglion were identified by their position on the anterior ventral side of the ganglion, by their size (60 to 80 μm diameter) and by their bioelectrical potentials. The resting potential of the Retzius cells ranged from −40 to −60 mV and the action potentials were generally between 20 and 30 mV and did not overshoot.

Intracellular recordings were made from the Retzius cells using high resistance glass microelectrodes (20 to 60 MOhms) filled with molar potassium acetate solution. The potentials were amplified through a

0007-1188/80/040711-07 $01.00

bridge/record system and displayed on a Tectronix 502A oscilloscope with permanent traces recorded on a Hewlett-Packard pen recorder.

Drugs were dissolved in leech Ringer and applied directly to the bath by a glass pipette over the preparation in a volume of 0.2 ml. The equipotent molar ratio (e.p.m.r.) for each analogue was calculated for at least 5 experiments from the ratio of the number of nmol producing comparable depolarizations. L-Glutamate was taken as the standard (1.0). If the e.p.m.r. was greater than one, then the compound was less potent than L-glutamate. Likewise, if the e.p.m.r. was less than one then the compound was more potent than L-glutamate. The following compounds were used in this study: L-glutamic acid, (BDH); D-glutamic acid, (±) 4-fluoroglutamic acid, DL-α-aminoadipic acid, DL-α-aminopimelic acid, L-α-aminosuberic acid, (Koch-Light laboratories); L-aspartic acid, γ-methyl ester glutamic acid, di-methyl ester glutamic acid, γ-mono-ethyl ester glutamic acid, di-ethyl ester glutamic acid, kainic acid, L-methionine-DL-sulphoximine, (Sigma); DL-homocysteic acid, L-cysteine-sulphinic acid, 3-amino-propylphosphonic acid, (Calbiochem); (±)β-methylglutamic acid, (+) γ-methylglutamic acid, (+) β-phenylglutamic acid, DL-1-amino-*cis*-1,3-dicarboxycyclohexane, DL-1-amino-*trans*-1,3-dicarboxycyclohexane (Dr C. G. Rasool); D-α-aminoadipic acid, D-α-aminopimelic acid, L-α-aminopimelic acid (Dr J.

Table 1 Mean relative potencies for a series of glutamate analogues and related compounds for their excitatory effects on Retzius cells of *Hirudo medicinalis*

Agonist	*e.p.m.r.*	(±) *s.e. mean*
Kainic acid	0.0056	0.001
Quisqualic acid	0.009	0.001
DL-*cis*-1-Amino-1,3-dicarboxycyclopentane	0.21	0.03
DL-Homocysteic acid	0.25	0.04
Ibotenic acid	0.95	0.16
L-Glutamic acid	1.0	
(±) 4-Fluoroglutamic acid	1.13	0.19
DL-α-Methylglutamic acid	1.3	0.14
(±) γ-Methylglutamic acid	2.38	0.76
DL-α-Aminoadipic acid	9.3	1.3
L-Aspartic acid	11.2	1.7
DL-*cis*-1-Amino-1,3-dicarboxycyclohexane	13.5	1.4
D-Glutamic acid	25.5	2.1
L-Cysteine sulphinic acid	68.0	13.6
D-Aspartic acid	112.3	19.0
DL-*trans*-1-Amino-1,3-dicarboxycyclopentane	105.4	17.8
(±) β-Methylglutamic acid	>100.0	
(±) β-Phenylglutamic acid	>100.0	
DL-N-Methylglutamic acid	>100.0	
DL-N-Methylaspartic acid	>100.0	
D-α-Aminoadipic acid	>100.0	
DL-α-Aminopimelic acid	>100.0	
L-α-Aminosuberic acid	>100.0	
γ-Methyl ester glutamic acid	>100.0	
Di-methyl ester glutamic acid	>100.0	
γ-Monoethyl ester glutamic acid	>100.0	
Di-ethyl ester glutamic acid	>100.0	
L-Methionine sulphoximine	>100.0	
DL-*trans*-1-Amino-1,3-dicarboxycyclohexane	>100.0	
D-α-Aminopimelic acid	Inactive	
3-Aminopropylphosphonic acid	Inactive	
L-β-(Erythro)hydroxy glutamic acid	Inactive	
N-methyl-D-aspartic acid	Inactive	

Results are mean of at least 5 experiments. The standard dose of glutamate was normally in the range 10–100 nmol.

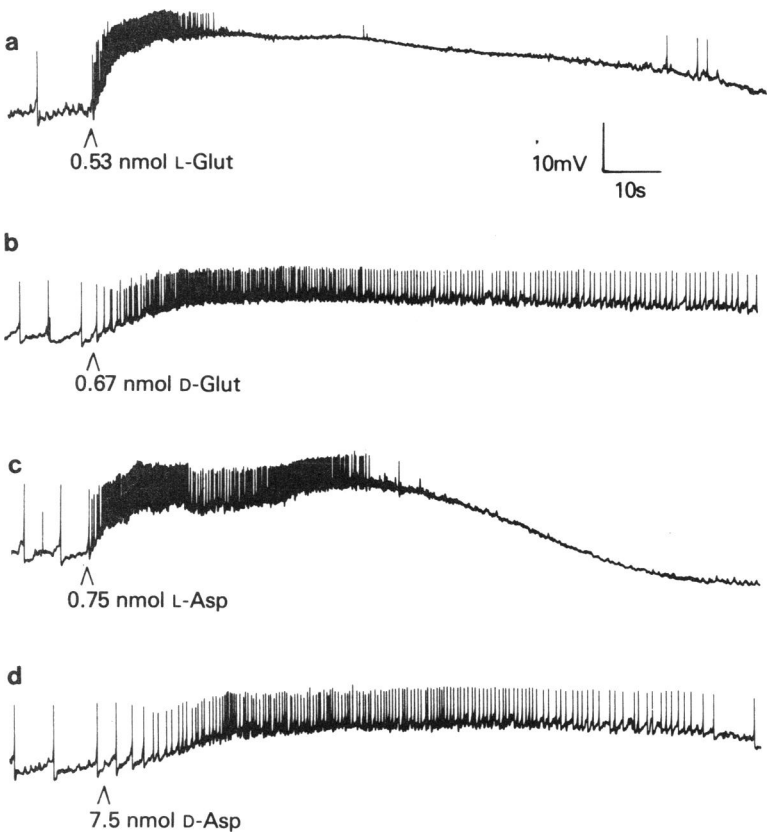

Figure 1 Traces of intracellular recordings from a leech Retzius cell to show the relative potencies of L-glutamate (a) compared to D-glutamate (b), with L-aspartate (c) and D-aspartate (d).

C. Watkins); quisqualic acid (Dr H. Shinozaki); L-β-(erythro)-hydroxy glutamic acid, (Dr H. Takeuchi); DL-1-amino-*cis*-1,3-dicarboxycyclopentane, DL-1-amino-*trans*-1,3-dicarboxycyclopentane, (Dr H. V. Wheal); and ibotenic acid (Prof. C. H. Eugester).

Results

L-Glutamic acid has an excitatory effect on the Retzius cells. This is a single response which involves an increase in permeability to both sodium and potassium ions (James & Walker, 1979). All the glutamate analogues were tested on the Retzius cells for glutamate-like activity, and their e.p.m.r's calculated from at least five different preparations. The results are summarized in Table 1. L-Glutamic acid was some 25 times more potent than the D-enantiomorph on the Retzius cells while L-aspartic acid was 11.2 times less potent than L-glutamate (Figure 1). There was no evidence for any interactions between L-aspartate and L-glutamate.

Substitution of a fluoro group on the α-carbon atom of the glutamate molecule had no significant effect on the activity of the glutamate molecule. Methylation of the α-carbon atom likewise had no effect on the potency of the molecule. However, methylation of the β-carbon atom considerably reduced the activity of the molecule such that there was no apparent depolarization of the Retzius cell, although there was some increase in the spontaneous firing rate. After treatment with β-methylglutamic acid the cells remained desensitized to subsequent standard doses of L-glutamate. The introduction of a hydroxyl group or a phenyl group on to the β-carbon atom of glutamic acid rendered the molecule at least a thousand times less active than L-glutamate. Both N-methyl-DL-glutamic acid and N-methyl-DL-aspartic acid had e.p.m.r.'s in excess of 100.

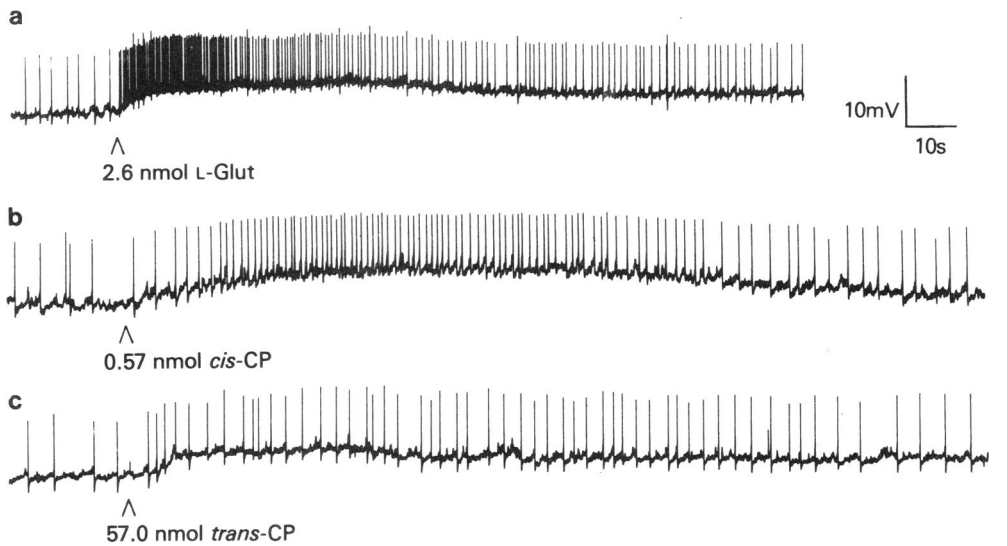

Figure 2 Traces of intracellular recordings from a leech Retzius cell to show the relative potencies of L-glutamate (a) with 1-amino-*cis*-1,3-dicarboxycyclopentane (*cis*-CP, b) and *trans* cyclopentane (*trans*-CP, c).

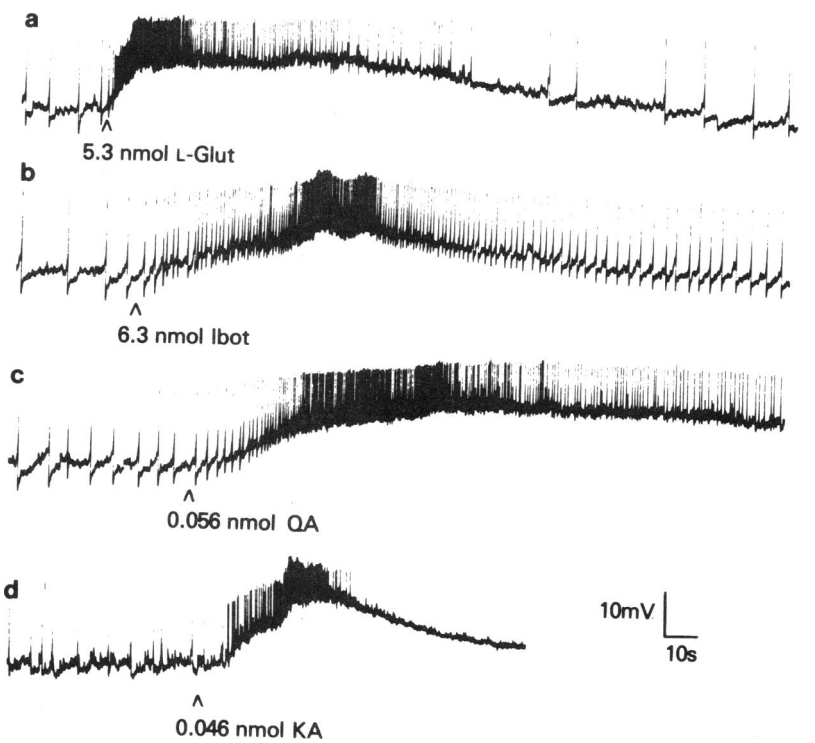

Figure 3 Traces of intracellular recordings from a leech Retzius cell to show the relative potencies of L-glutamate (a) compared to ibotenic acid (Ibot, b), quisqualic acid (QA, c) and kainic acid (KA, d).

DL-α-Aminoadipic acid had an e.p.m.r. of 9.3 being less active than L-glutamate at the Retzius cell. The D-enantiomorph of this compound was much less active than the racemic mixture with an e.p.m.r. greater than a thousand and had no apparent blocking action against L-glutamate. The D-enantiomorph of α-aminopimelic acid proved to be completely inactive at the Retzius cell.

DL-Homocysteic acid had an e.p.m.r. of 0.25, being more potent than L-glutamate, whilst L-cysteine sulphinic acid and 3-amino-propylphosphonic acid were completely inactive.

Ibotenic acid was approximately equipotent with L-glutamate and DL-1-amino-*cis*-1,3-dicarboxycyclopentane was some 5 times more potent, whilst the *trans* form was at least a hundred times less potent than L-glutamate (Figure 2). DL-1-Amino-*cis*-1,3-dicarboxycyclohexane was approximately 10 times less potent than L-glutamate and the *trans* was more than a thousand times less potent. Kainic acid and quisqualic acid were both very much more potent than L-glutamate (Figure 3).

L-Methionine-DL-sulphoximine, γ-methyl ester glutamic acid, di-methyl ester glutamic acid, γ-mono ethyl ester glutamic acid and di-ethyl ester glutamic acid showed neither antagonistic nor agonistic activity on the leech Retzius cells.

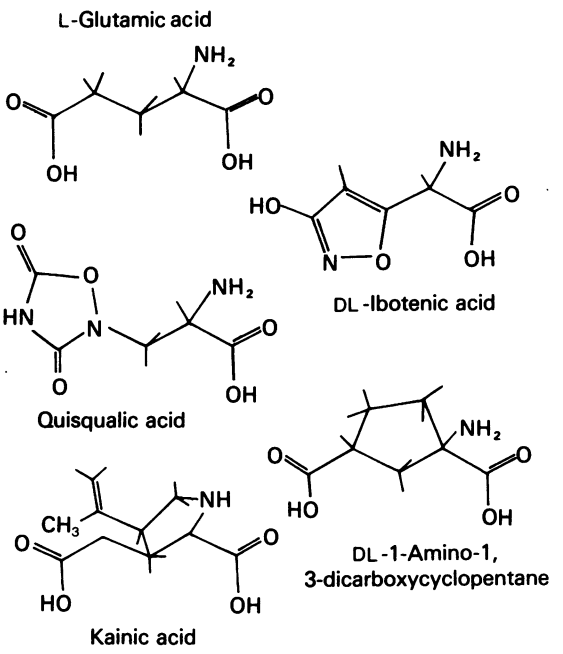

Figure 4 Structures of the most active analogues used in this study.

Discussion

This structure-activity study shows that the glutamate receptor at the leech Retzius cell has stereospecificity for the L-isomer of glutamic acid rather than the D-isomer which is about 25 times less active than the L-form. This has also been shown to be true of glutamate receptors at the crayfish and locust neuromuscular junction (Takeuchi & Takeuchi, 1964; Clements & May, 1974) and at the crayfish vas deferens (Murdock, 1971). Decreasing the length of the carbon chain as in L- and D-aspartic acid or lengthening the carbon chain by 1,2 or 3 carbon atoms causes a decrease in the excitatory activity at the Retzius cell, an effect also exhibited by glutamate receptors of *Helix aspersa* (Piggott, Kerkut & Walker, 1975) and the receptors of the locust neuromuscular junction (Clements & May, 1974).

Evidence suggests that there are two types of glutamic receptors, 'glutamate-' and 'aspartate'-preferring receptors (Morgan *et al.*, 1972; Johnston *et al.*, 1974). These 'glutamate'-preferring receptors have a high affinity for glutamate in an extended conformation and hence for the conformationally restricted analogues ibotenic acid and kainic acid (Figure 4) (Lea & Usherwood, 1973; McCulloch *et al.*, 1974; Johnston *et al.*, 1974; Buu, Puil & Van Gelder, 1975), whilst 'aspartate'-preferring receptors have a high affinity for glutamate in a folded conformation resembling aspartic acid, and so have a preference for aspartic acid (Johnston *et al.*, 1974; McCulloch, *et al.*, 1974).

At the leech Retzius cell, ibotenic acid is approximately equipotent with L-glutamate while DL-1-amino-*cis*-1,3-dicarboxycyclopentane (Figure 4) is 5 times more potent than L-glutamate. Computer analysis has indicated that both DL-1-amino-*cis*-1,3-dicarboxycyclopentane and ibotenate have relatively fixed and similar binding conformations (McLennan & Wheal, 1978). Aspartate is not able to assume this conformation. In the rat thalamus DL-1-amino-*cis*-1,3-dicarboxycyclopentane and ibotenic acid are respectively 9.74 and 7.45 times more potent than L-glutamate (Hall, Hicks, McLennan, Richardson & Wheal, 1979). Thus, the potency ratio between DL-1-amino-*cis*-1,3-dicarboxycyclopentane and L-glutamate are comparable in the two preparations. The potency of DL-1-amino-*cis*-1,3-dicarboxycyclopentane on the leech Retzius cell provides further evidence that glutamate is acting in an extended conformation. Kainic acid is at least a hundred times more potent than L-glutamate at the Retzius cell whilst aspartic acid is some 10 times less potent, and N-methyl-D-aspartic acid is inactive. This would suggest that the amino acid receptor at the leech Retzius cell is 'glutamate'-preferring.

Computer calculations show that DL-1amino-*cis*-1,3-dicarboxycyclopentane analogue approaches the extended conformation of L-glutamate. Previously published data (Hall *et al.*, 1979) show that the distances between the amino group and the distal carboxyl group of 1-amino-*cis*-1,3-dicarboxycyclopentane and 1-amino-*trans*-1,3-dicarboxycyclopentane are different, being 4.64 Å and 3.35 Å respectively. This difference may explain the low activity of the *trans* isomer. The low activity of the *cis* and *trans* 1-amino-1,3-dicarboxycyclohexane analogues may be explained by the uncertainty of their configuration together with possible steric effects. Johnston *et al.*, (1974) suggested that these analogues are active in the less stable 'boat' form.

From the relatively poor responses of L-aspartate and 1-amino-*trans*-1,3-dicarboxycyclopentane in this study, it could be suggested that the glutamate receptors in the leech are more specific than those reported for the rat central nervous system (Hall *et al.*, 1979).

Recently, however, evidence has been presented to suggest that ibotenic acid may have an effect on spinal interneurones, independent of the glutamate excitatory receptors (MacDonald & Nistri, 1977) and may not, therefore, be a specific agonist as previously thought. Ibotenic acid also appears to activate the extrajunctional receptor on the insect muscle rather than the junctional glutamate receptors (Lea & Usherwood, 1973) but appears to be inactive on glutamate receptors at crustacean neuromuscular junction (Wheal & Kerkut, 1973). It has been suggested by Johnston *et al.* (1974), that kainic acid may also have an indirect effect, for whilst kainic acid has a ring component similar to glutamate, it also has a side chain which may interact with proteolytic sites near to the glutamate receptor which could account for the high affinity of the molecule for the receptor. Johnston *et al.* (1974), found that hydrogenation of the double bond in the side chain of kainic acid reduces the activity of the compound on cat Renshaw cells. Our preliminary experiments, however, with di-hydro kainic acid fail to support this hypothesis, the compound being equipotent with kainic acid and thus more potent than L-glutamate. Thus, further work is required to try to determine the reason for the high potency of kainic acid at this site.

There are clearly differences between the leech Retzius cell glutamate receptors and those of *Helix* neurones. In the latter case, kainic acid is always less potent than glutamate (Walker, 1976). However, quisqualic acid was found to be more potent than glutamate on certain *Helix* glutamate receptors, in some cases being 100 to 1000 times more potent than L-glutamate. Quisqualic acid has also been found to be more potent than glutamate on crayfish muscle (Shinozaki & Shibuya, 1974). Thus, while in the present study quisqualic and kainic acids are approximately equipotent, this is by no means the general rule among invertebrate preparations.

A number of compounds have been suggested as glutamate/aspartate antagonists: L-methionine sulphoximine, methyl and ethyl esters of glutamic acid; D-2-aminopimelic acid, D-2-aminoadipic acid and HA-966 (Haldeman, Huffman, Marshall & Itchennan, 1972; Haldeman & McLennan, 1972; Lowagie & Gerschenfeld, 1974; Kerkut, Piggott & Walker, 1975; Biscoe, Evans, Francis, Martin, Watkins, Davies & Dray, 1977a. b; Evans & Watkins, 1978). However, preliminary studies on the leech Retzius cell glutamate receptor have failed to find an effective glutamate antagonist though both β-methyl-glutamate and DL-2-aminopimelic acid in addition to being weak agonists desensitized the leech glutamate receptor to glutamate.

The present study provides some evidence for an excitatory glutamate receptor with a conformational requirement for glutamate in an extended form. As yet, there is no evidence for glutamate as an excitatory transmitter in the leech central nervous system and further studies are required on this point.

References

BUU, N.T., PUIL, E. and VAN GELDER N.M. (1976). Receptors for amino acids in excitable tissues. *Gen. Pharmac.*, **1**, 5–14.

BISCOE, T.J., DAVIES, J., DRAY A., EVANS, R.H., FRANCIS, A.A., MARTIN, M.R. & WATKINS, J.C. (1977). Depression of synaptic excitation and of amino acid induced excitatory responses of spinal neurones by D-α-aminoadipate, α, ϵ-diaminopimelic acid and HA-966. *Eur. J. Pharmac.*, **45**, 315–316.

BISCOE, T.N., EVANS, R.H., FRANCIS, A.A., MARTIN, M.R., WATKINS, J.C., DAVIES, J. & DRAY, A. (1977). D-α-amino adipate as a selective antagonist of amino acid induced and synaptic excitation of mammalian spinal cord *Nature* (*Lond.*), **270**, 743–745.

CLEMENTS, A.N. & MAY, T.E. (1974). Pharmacological studies on a locust neuromuscular preparation. *J. exp. Biol.*, **61**, 421–442.

CURTIS, D.R. & JOHNSTON, G.A.R. (1974). Amino acid transmitters in mammalian CNS. *Ergebn. Physiol.* **69**, 97–188.

EVANS, R.H. & WATKINS, J.C. (1978). Dual sites for antagonism of excitatory amino acids on central neurones. *J. Physiol.*, **277**, 57P.

GERSCHENFELD, H.M. (1973). Chemical transmission in invertebrate CNS and neuromuscular junctions. *Physiol. Rev.*, **53**, 1–119.

HALL, J.G., HICKS, T.P., MCLENNAN, H., RICHARDSON T.L. & WHEAL, H.V. (1979). The excitation of mammalian

central neurones by amino acids. *J. Physiol.*, **286**, 29–39.

HALDEMAN, S., HUFFMAN, R.D., MARSHALL, K.C. & MCLENNAN, H. (1972). The antagonism of the glutamate induced and synaptic excitation of thalamic neurones. *Brain Res.*, **39**, 419–425.

HALDEMAN, S. & MCLENNAN, H. (1972). The antagonism of the glutamate induced and synaptic excitation of thalamic neurones. *Brain Res.*, **45**, 393–400.

JAMES, V.A. & WALKER, R.J. (1978). Structure-activity studies on an excitatory glutamate receptor of leech neurones. *Br. J. Pharmac.*, **62**, 432–433P.

JAMES, V.A. & WALKER, R.J. (1979). The ionic mechanism responsible for glutamate excitation of leech Retzius cells. *Comp. Biochem. Physiol.*, **64C**, 261–266.

JOHNSTON, G.A.R., CURTIS, D.R., DAVIES, J. & MCCULLOCH, R.M. (1974). Spinal interneurones excitation by conformationally restricted analogues of L-glutamic acid. *Nature (Lond.)*, **248**, 804–805.

KERKUT, G.A., PIGGOTT, S.M. & WALKER, R.J. (1975). The antagonist effect of alpha aminopimelic acid on glutamate-induced inhibitions of *Helix* neurones. *Brain Res.*, **86**, 139–143.

LEA, T.J. & USHERWOOD, P.N.R. (1973). The site of action of ibotenic acid and the identification of two populations of glutamate receptors on insect muscle fibres. *Comp. gen. Pharmac.*, **4**, 333–350.

LOWAGIE, C. & GERSCHENFELD, H.M. (1974). Glutamic acid antagonists at a crayfish neuromuscular junction. *Nature (Lond.)*, **248**, 533–535.

MACDONALD, J.F. & NISTRI, A. (1977). Actions of microiontophoretically applied ibotenate on cat spinal interneurones. *Can. J. Physiol. Pharmac.*, **55**, 965–967.

MCCULLOCH, R.M., JOHNSTON, G.AA.R., GAME, C.J.A. & CURTIS, D.R. (1974). The differential sensitivity of spinal interneurones and Renshaw cells to kainate and N-methyl-D-aspartic acid. *Exp. Brain Res.*, **21**, 518–519.

MCLENNAN, H. & WHEAL, H.V. (1978). A synthetic, conformationally restricted analogue of L-glutamic acid which acts as a powerful neuronal excitant. *Neurosci. Letts*, **8**, 51–54.

MORGAN, R., VRBOVA, G. & WOLSTENCROFT, J.H. (1972). Correlation between retinal input to lateral geniculate neurones and their relative response to glutamate and aspartate. *J. Physiol.*, **224**, 41–42P.

MURDOCK, L.L. (1971). Crayfish vas deferens: Contractions in response to L-glutamate and gamma-aminobutyric acid. *Comp. gen. Pharmac.*, **2**, 93–98.

NICHOLLS, J.G. & KUFFLER, S.W. (1964). Extracellular space as a pathway for exchange between blood and neurones in the central nervous system of the leech: ionic composition of glial cells and neurones. *J. Neurophysiol.*, **27**, 645–671.

PIGGOTT, S.M., KERKUT, G.A. & WALKER, R.J. (1975). Structure activity studies on glutamate receptor sites of 3 identifiable neurones in the sub-oesophageal ganglia of *Helix aspersa*. *Comp. Biochem. Physiol.*, **51C**, 91–100.

SHINOZAKI, H. & SHIBUYA, I. (1974). A new potent excitant, Quisqulic acid, effects on crayfish neuromuscular junction. *Neuropharmac.*, **13**, 665–672.

TAKEUCHI, A. & TAKEUCHI, N. (1964). The effect on crayfish muscle of iontophoretically applied glutamate. *J. Physiol.*, **170**, 296–317.

USHERWOOD, P.N.R. (1977). Neuromuscular transmission in insects. In *Identified Neurons and Behaviour of Arthropods*. ed. Hoyle, G., pp. 31–48. New York: Plenum.

USHERWOOD, P.N.R. (1978). Amino acids as transmitters. *Adv. comp. Physiol. Biochem.*, **7**, 225–309.

WALKER, R.J. (1976). The action of kainic acid and quisqualic acid on the glutamate receptors of three identifiable neurones from the brain of *Helix aspersa*. *Comp. Biochem. Physiol.*, **55C**, 61–67.

WHEAL, H.V. & KERKUT, G.A. (1973). Structure activity studies on the excitatory receptor of the crustacean neuromuscular junction. *Comp. Biochem. Physiol.*, **53C**, 51–55.

(*Received April* 30, 1979.
Revised August 7, 1979.)