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Inhibition of the glutamate transporter and glial enzymes in rat striatum by the gliotoxin, αaminoadipate

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1 The effect of the gliotoxic analogue of glutamate, αaminoadipate, on the high affinity transport of D-[^{3}H]-aspartate into a crude striatal P_2 preparation, and on the activity of two enzymes of which glutamate is the substrate has been examined.

2 The L-isomer of αaminoadipate competitively inhibited the transport protein, with a K_i value of 192 μM, whereas the D-isomer of αaminoadipate was ineffective. The potent convulsant, L-methionine-*S*-sulphoximine, was also without effect on the activity of the gluatmate transport protein.

3 L-αAminoadipate was a competitive inhibitor of both glutamine synthetase, and γ-glutamylcysteine synthetase, with K_i values of 209 μM and 7 mM respectively. Once again, the D-isomer of αaminoadipate was a far weaker inhibitor of either enzyme.

4 The results are discussed in terms of the mechanism of action of αaminoadipate in causing toxicity of glial cells.

Keywords: glutamate transport; glutamine synthetase; γ-glutamylcysteine synthetase; αaminoadipate; glial cell; toxicity

Introduction

Several analogues of the excitatory transmitter, L-glutamate, have been shown to be extremely potent neurotoxins after either direct injection into the brain, or during incubation of tissue slices or cell cultures *in vitro* (McBean & Roberts, 1985; Meldrum & Garthwaite, 1990). αAminoadipate (αAA) is a structural analogue of glutamate which, in contrast to most other glutamate-like compounds, is toxic to glial cells. Injection of DL-αAA into the retina of rats results in marked, but transient, morphological damage to Muller (glial) cells, without any change in the morphological integrity of the surrounding neurones (Olney *et al.*, 1971). Similarly, intracerebral application of the compound into the striatum of adult rats causes a significant reduction in the activity of the glial enzyme, glutamine synthetase, at 6 h after the injection, with a return to normal levels of activity by 24 h after the lesion, whereas neuronal cells are unaffected by this treatment at either time (McBean, 1990).

Studies on the stereospecificity of the effects of αAA have shown that the 'active component', in terms of its gliotoxicity, is the L-isomer (Olney *et al.*, 1980), which is also a weak neuroexcitant (McLennan & Hall, 1978). The D-isomer, on the otherhand, which is an N-methyl-D-aspartate (NMDA) receptor antagonist, is not gliotoxic and may actually be neuroprotective (Olney *et al.*, 1980).

Little is known of how L-αAA causes toxicity to glial cells, but since this compound is the most effective gliotoxin identified (Olney *et al.*, 1971), it is unlikely that excitation of neuronal glutamate receptors alone could account for its toxic potency. One component of the action of L-αAA may be an inhibition of cystine transport into glial cells, which could contribute to a reduction in the quantity of glutathione available for protection of tissue against free radical-induced cell damage (Kato *et al.*, 1993). However, there are several other aspects of glutamate metabolism in glial cells which, if disrupted by L-αAA, could provide an alternative mechanism for causing the toxic effects of this compound. This paper describes a series of experiments in which the effects of both the racemate and separate isomers of αAA were assessed on the activity of the plasma-membrane glutamate transporter, and on two enzymes, glutamine synthetase and γ-glutamylcysteine synthetase, both of which are associated with the glial cell metabolism of glutamate (Meister & Tate, 1979; Norenberg & Martinez-Hernandez, 1979). In the case of the glutamate transporter, the non-metabolizable analogue of glutamate, D-aspartate, was used to measure the rate of glutamate transport.

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Methods

High-affinity sodium-dependent transport of D-[^{3}H]-aspartate into crude striatal synaptosomes

Rats were killed by cervical dislocation, and the brains rapidly removed and placed on ice. A 5% (w/v) homogenate of the striatum was prepared using 0.32 M sucrose in 50 mM Tris-HCl, pH 7.4. Following centrifugation at 3,200 r.p.m. for 5 min, the supernatant was removed and re-centrifuged at 12,000 r.p.m. for 12 min. The pellet was washed once in Tris-sucrose, and re-precipitated by centrifugation as before at 12,000 r.p.m. The final pellet (P_2) was re-suspended in Tris-sucrose and maintained on ice until the transport assay.

D-[^{3}H]-aspartate transport

A tube containing 975 μl of Krebs bicarbonate medium (contents, in mM: NaCl 109.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 11.5), pH 7.4 and 25 μl P_2 was pre-incubated at 25°C for 5 min and the reaction started by the addition of 25 μl D-[^{3}H]-aspartate (final specific activity 0.125 μCi nmol^{-1}). The concentration of D-aspartate used ranged from 0.39 μM to 1.95 μM (experiments prior to this study having established that the sodium-dependent high affinity transport of D-aspartate had a K_m of 10 μM, and a V_{max} of 35 nmol mg^{-1} protein 4 min^{-1}). The incubation was continued for 4 min and was terminated by centrifugation at 10,000 r.p.m. in a microcentrifuge. The pellet was washed once in Tris-sucrose and extracted overnight with 200 μl sodium dodecyl sulphate. The quantity of radioactivity present was determined by liquid scintillation spectroscopy. Sodium-free Krebs medium was used to assess the sodium-dependency of the transport.

Glutamine synthetase

Rat striata were homogenized in 0.1 M sodium acetate, pH 7.4, centrifuged at 1,200 *g*, and the supernatant used for the assay. The γ-glutamyl-transfer activity of the enzyme was measured according to the method of Wellner & Meister (1966), in which 150 mM glutamine (unless otherwise stated) was added as substrate, in the presence of 0.1 mM ATP, 200 mM hydroxylamine and 33 mM $MnCl_2$. The quantity of γ-glutamylmono-hydroxamate (GHA) formed at 37°C over 20 min was determined spectrophotometrically at 500 nm.

γ-Glutamylcysteine synthetase

Samples of whole rat brain were homogenized in 9 volumes of ice-cold 0.15 M KCl, containing 0.5 mM mercaptoethanol and 1 mM $MgCl_2$, and then subjected to partial purification by ammonium sulphate fractionation. The activity of the enzyme was determined at 37°C during 30 min incubation of 100 μl samples of the purified homogenate by the method of Richman *et al.* (1973), using 2 mM glutamate (unless otherwise stated) and 10 mM α-aminobutyrate (replacing cysteine) as substrates in the presence of 20 mM $MgCl_2$ and 5 mM ATP in a final volume of 0.5 ml. Incubation of the samples was continued for 30 min at 37°C and terminated by the addition of 0.5 ml 10% trichloroacetic acid. Following centrifugation, the quantity of inorganic phosphate in the supernatant was determined by colourimetric assay. Protein determinations were performed on each set of tissue preparation by the method of Markwell *et al.* (1978).

The results in each table are expressed as the mean ± s.e.mean of the number of observations indicated. Statistical analysis of each set of data was by Student's unpaired *t* test, and the difference between means was regarded as significant at *P* values of less than 0.05.

L-Methionine-*S*-sulphoximine and αaminoadipate were purchased from the Sigma Chemical Co., Poole, Dorset. D-[^{3}H]-aspartate (specific activity 25 Ci mmol^{-1}) was provided by Radiochemical Centre, Amersham, Bucks. All other chemicals and reagents were bought from either BDH, Ltd., England, or Sigma.

Results

The effects of αaminoadipate on D-[^{3}H]-aspartate transport

Samples of crude P_2 were incubated at 25°C for 4 min in the presence of 0.39 μM D-[^{3}H]-aspartate. Addition of L-αAA to the incubation medium resulted in a dose-dependent reduction in the rate of sodium-dependent D-[^{3}H]-aspartate transport from 1.53 ± 0.17 nmol mg^{-1} protein 4 min^{-1} to 1.16 ± 0.12 nmol mg^{-1} protein 4 min^{-1} with 100 μM L-αAA, and 0.53 ± 0.07 nmol mg^{-1} protein 4 min^{-1} with 500 μM L-αAA, as shown in Table 1. DL-αAA was a weaker inhibitor of D-[^{3}H]-aspartate transport, since the effect of adding 500 μM DL-αAA to the incubation mixture resulted in only a 41% reduction in the rate of D-[^{3}H]-aspartate transport (0.90 ± 0.14 nmol mg^{-1} protein 4 min^{-1}). The D-isomer of αAA showed a minimal effect on the high-affinity uptake of D-[^{3}H]-aspartate, and L-methionine-*S*-sulphoximine (MSO) was also ineffective as an inhibitor of the glutamate transporter. L-αAA proved to be a competitive inhibitor of the transport protein, as shown in Figure 1, with a K_i value of 192 μM.

The effect of αaminoadipate on the acitivity of glutamine synthetase in striatal homogenates

Once again, the L-isomer of αAA was the more potent inhibitor of glutamine synthetase, and a dose-dependent inhibition of the enzyme was observed following co-incubation of the substrate with L-αAA (Table 2)·. Although D-αAA was markedly less effective than L-αAA, inhibition of the activity of glutamine synthetase was observed, at high concentrations, as shown in Table 2, where 1 mM D-αAA reduced the activity of the enzyme to 55% control (15.4 ± 1.6

Table 1 Inhibition of D-[^{3}H]-aspartate transport into a striatal P_2 preparation by αaminoadipate (αAA) and L-methionine-*S*-sulphoximine (MSO)

Inhibitor	*Concentration* (μM)	*Transport* (nmol mg^{-1} protein 4 min^{-1})	*(% of control)*
Control		1.53 ± 0.17	
L-αAA	100	1.16 ± 0.12	76
	500	0.53 ± 0.07	37**
DL-αAA	100	1.04 ± 0.09	78
	500	0.90 ± 0.14	59**
D-αAA	100	1.26 ± 0.11	83
	500	1.19 ± 0.14	78
MSO	100	1.52 ± 0.16	99
	500	1.35 ± 0.17	88

Mean ± s.e.mean of at least 4 independent observations, measured in triplicate. *$P<0.05$ vs control; **$P<0.01$ vs control.

Table 2 Inhibition of striatal glutamine synthetase by αaminoadipate (αAA) and L-methionine-*S*-sulphoximine (MSO)

Inhibitor	*Concentration* (μM)	*Activity* (nmol GHA mg^{-1} protein min^{-1})	*(% of control)*
Control		27.7 ± 2.6	
L-αAA	200	14.5 ± 1.2	52**
	1000	7.2 ± 0.6	26**
DL-αAA	200	15.4 ± 1.6	55**
	1000	9.6 ± 0.6	35**
D-αAA	200	26.0 ± 2.4	94
	1000	15.4 ± 1.6	55**
MSO	200	3.2 ± 0.4	11***
	1000	0.7 ± 0.2	3***

Mean ± s.e.mean of at least 3 independent observations, measured in triplicate. *$P<0.02$ vs control; **$P<0.01$ vs control; ***$P<0.001$ vs control.

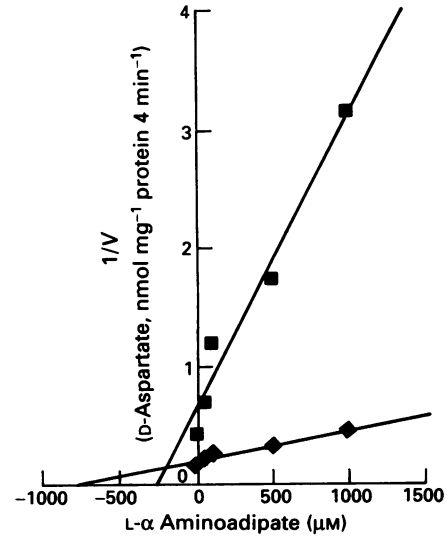

Figure 1 Dixon plot showing inhibition of D-[^{3}H]-aspartate transport by L-αaminoadipate, at 0.39 μM (■) and 1.95 μM aspartate (◆). The results are the mean of at least 3 independent observations, measured in triplicate.

nmol GHA formed mg^{-1} protein min^{-1}). As expected, MSO was an extremely potent inhibitor of the enzyme, and only 11% of the control activity of the enzyme remained after incubation with 200 μM MSO.

L-αAA was a competitive inhibitior of glutamine synthetase, with respect to the substrate, glutamine, with a K_i value of 109 μM, as shown in Figure 2.

Inhibition of γ-glutamylcysteine synthetase by L-αaminoadipate and L-methionine-S-sulphoximine

αAA proved to be a weak competitive inhibitor of γ-glutamylcysteine synthetase (Table 3), although, once again, the L-isomer of αAA was the most potent form of the compound: in the presence of 10 mM L-αAA the activity of the enzyme was reduced to 67% control (0.179 ± 0.013 μmol Pi mg^{-1} protein min^{-1}). A 10 mM concentration of MSO gave an almost total inhibition in the activity of the enzyme. Kinetic analysis (Figure 3) indicated that L-αAA competes with glutamate, as does MSO, but the K_i for inhibition of this enzyme by L-αAA is considerably higher (7 mM) than that for inhibition of glutamine synthetase. Inhibition of γ-glutamylcysteine synthetase by L-αAA was independent of any variation in the concentration of the second substrate, αaminobutyrate (results not shown).

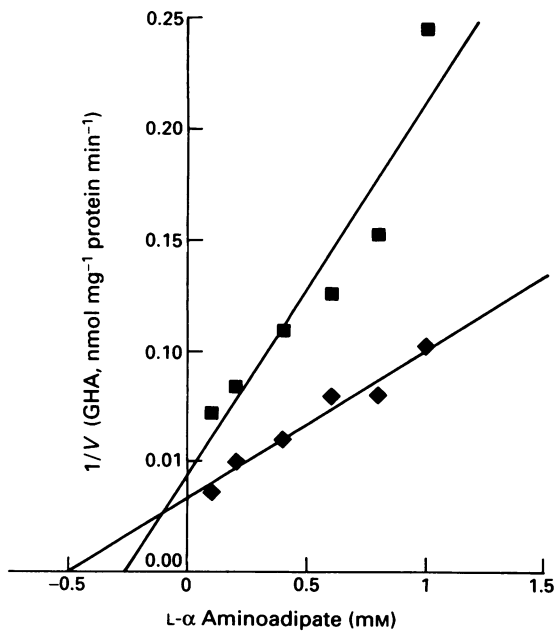

Figure 2 Graph showing competitive inhibition of glutamine synthetase activity by L-αaminoadipate, at 200 μM (■) and 300 μM (◆) glutamine. Each point is the mean of at least 3 independent observations, measured in duplicate.

Table 3 Inhibition of γ-glutamylcysteine synthetase by L-αaminoadipate and L-methionine-*S*-sulphoximine

Inhibitor	*Concentration* (mM)	*Activity* (μmol Pi mg^{-1} protein 30 min^{-1})	*(% of control)*
None		0.264 ± 0.017	
L-αAA	2.5	0.234 ± 0.012	89
L-αAA	10	0.179 ± 0.013	67*
D-αAA	10	0.273 ± 0.014	103
MSO	10	0.023 ± 0.004	8.7**

Mean ± s.e.mean of 4 independent observations, measured in duplicate. *$P<0.02$; **$P<0.001$.

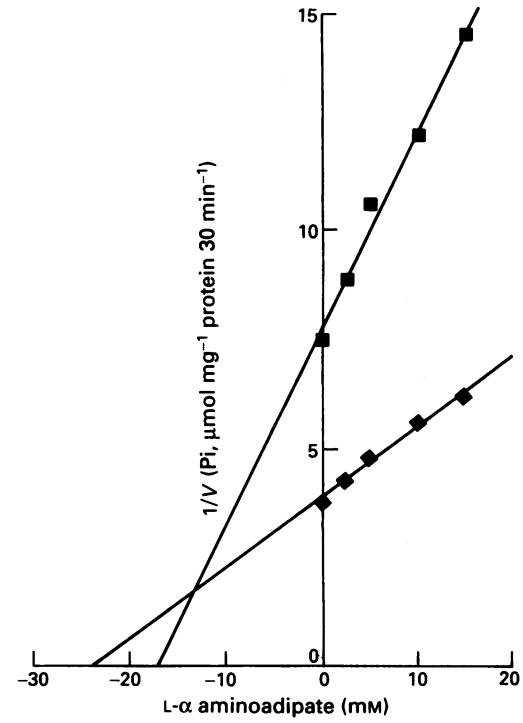

Figure 3 Dixon plot showing inhibition of γ-glutamylcysteine synthetase by L-αaminoadipate, at 0.5 mM (■) and 2.0 mM glutamate (◆). Each point is the mean of 2 independent observations, measured in duplicate.

Discussion

The results described in this paper clarify several aspects of the mechanism of gliotoxicity by αAA. Firstly, in each of the parameters of glutamate transport and metabolism tested, it is the L-isomer which is by far the most active form of αAA. The same stereospecificity of αAA was reported by Olney *et al.* (1980), who presented evidence showing that the L-isomer was the most toxic form of αAA to retinal Muller cells after application *in vivo*, and a similar order of potency of the various forms of αAA was observed more recently by Kato *et al.* (1993) in experiments in which the effects of αAA on the glutathione content of carp retina was measured.

The second aspect of the toxicity of αAA which is clarified by the results presented in this paper is the question of the glial-cell specificity. As the L-isomer of αAA is no more than a weak excitant of neuronal glutamate receptors (McLennan & Hall, 1978), the toxicity of this compound is much more likely to be mediated by a direct effect on glial cells themselves. The glutamate high-affinity, sodium-dependent, transporter is located on both neuronal and glial cells in the brain (Drejer *et al.*, 1982), and is responsible for the rapid removal of glutamate from the synaptic cleft following activation of excitatory pathways and release of the neurotransmitter (Nicholls & Attwell, 1990). Both immunocytochemical localization experiments and *in situ* hybridization studies with mRNA coding for GLAST and GLT-1 glutamate transporters have revealed a glial localization for these transport proteins (Danbolt *et al.*, 1992; Pines *et al.*, 1992; Storck *et al.*, 1992) and recent evidence has shown that at least one of these transporters may be regulated physiologically by protein kinase C-mediated phosphorylation of the protein (Casado *et al.*, 1993). Studies on neuronal and glial cells in culture have indicated that the glial site of transport is predominant (Drejer *et al.*, 1982), which could explain why L-αAA, acting primarily through inhibition of the glutamate transporter, would initially cause a disruption of glutamatergic activity in glial cells. The same rationale has been offered by Kato *et al.* (1993) to explain the glial-cell

specificity of L-αAA in inhibiting the cystine/glutamate exchanger in retinal Muller cells, and similarly, the potent glycolytic inhibitor, fluorocitrate, acts preferentially on glial cells at low concentrations (Paulsen *et al.*, 1987). It has been noted previously (McBean, 1990) that during incubation of striatal slices with DL-αAA, at high concentrations of the toxin (for example, superfusion for 40 min with 1 mM), neuronal cells also become affected and die. Indeed, one of the most potent inhibitors of glutamate transport known, DL-*threo*-3(OH) aspartate, caused neuronal degeneration in the striatum after intracerebral injection (McBean & Roberts, 1985). There are two possible reasons for this spread of toxicity to neuronal cells with high concentrations of DL-αAA: firstly, with increasing concentrations of the toxin, neuronal transport of glutamate would also be inhibited, and secondly, the eventual outcome of prolonged inhibition of either glial or neuronal transport would be an elevation in glutamate concentrations in the synaptic cleft, activation of post-synaptic receptors, and a process of disruption of glutamatergic function akin to that proposed to occur in ischaemia (Nicholls & Attwell, 1990).

Although the question of whether L-αAA is a substrate, as well as an inhibitor, of the high-affinity glutamate transport has not been addressed directly in this series of experiments, previous work by Huck *et al.* (1984) had indicated that in cultures at least, L-αAA is actively accumulated into glial cells before its toxic effects are observed. It is highly probable, therefore, that L-αAA gains access into striatal glial cells by means of the glutamate transporter.

As in the case of the glutamate transport protein, L-αAA is also the active isomer which competitively inhibits the activity of glutamine synthetase, with a comparable K_i value to that of the inhibition of transport, although the degree of inhibition of this enzyme is greater at a given concentration of the toxin than for the glutamate transporter. In this case, however, the D-isomer does have some inhibitory activity, and this is in agreement with some of the original work on glutamine synthetase by Wellner *et al.* (1966), in which it was shown that both the L- and D-isomers of αAA are not only inhibitors, but also substrates of the enzyme. This observation may explain the third feature of αAA toxicity: the transience of αAA effects. It has been noted that in experiments *in vivo* (Olney *et al.*, 1980; McBean, 1990), morphological changes to glial cells, and a reduction in the activity of the glial marker enzyme, glutamine synthetase, are apparent at 6, but not 24 h, after intraretinal or intrastriatal injections, respectively. As αAA is a substrate for glutamine synthetase, it is reasonable to assume that this would account for the gradual decline in its toxic potency.

To what extent, then, does inhibition of glutamine synthetase by L-αAA contribute to the toxicity of this compound? MSO is a much more potent inhibitor of glutamine synthetase than is either isomer of αAA, and is, moreover, an irreversible inhibitor of this enzyme (Cooper *et al.*, 1983). Intracerebral injection of MSO is well-known to cause convulsions, which are due to an elevation in ammonia levels, rather than an alteration in glutamate concentration *per se* (Cooper *et al.*, 1983). DL-αAA, on the otherhand, which does not cause convulsions after intra-striatal injection (McBean, 1990) would not, presumably, have the same disruptive influence on ammonia levels, because it is a substrate of the enzyme. The similarity between the K_i values of both the transport of D-[^{3}H]-aspartate and this enzyme would imply that the concentration of L-αAA inside the glial cell would be sufficient to inhibit the enzyme, but the precise implication of this, as regards a disruption in glutamate metabolism, needs further investigation.

It is not surprising that L-αAA also inhibits γ-glutamylcysteine synthetase in addition to glutamine synthetase, as there are many mechanistic similarities between these two enzymes: γ-glutamylcysteine synthetase is, like glutamine synthetase, found in glial cells (Meister & Tate, 1976) and both reactions proceed via an enzyme-bound γ-glutamylphosphate intermediate, before entry of the second substrate (ammonia for glutamine synthetase; and cysteine for γ-glutamylcysteine synthetase). However, it is unlikely that the direct inhibition of γ-glutamylcysteine synthetase by L-αAA contributes much to the overall toxicity of this compound in glial cells, for the following reasons: firstly, L-αAA is a far less effective inhibitor of γ-glutamylcysteine synthetase than of glutamine synthetase, as the differences in the magnitude of the K_i for each enzyme testifies (see Figures 2 and 3). Secondly, it is the intracellular concentration of cysteine that regulates the activity of the enzyme, not glutamate, with which L-αAA competes (Meister & Tate, 1976).

γ-Glutamylcysteine synthetase is the rate-limiting enzyme of the γ-glutamyl cycle, which is responsible for maintaining glutathione levels in cells (Meister & Tate, 1976). There is currently much interest in the role of glutathione in the brain in preventing damage to cells by oxidative stress (Pellmar *et al.*, 1993). It has previously been shown that L-αAA in the retina leads to a reduction in GSH concentration (Kato *et al.*, 1993), and the same observation has been made using incubation of striatal slices with L-αAA (McBean, unpublished results). Inhibition of a cystine transporter by L-αAA may provide a possible mechanism for depletion of glutathione, but given the very slow rate of this transporter (V_{max}: 8.3 nmol 5 mg^{-1} tissue 15 min^{-1}; Kato *et al.*, 1993), it is debatable whether the supply of cysteine for the synthesis of glutathione would be enormously depleted by inhibition of this transporter alone. γ-Glutamyltranspeptidase activity, for instance, is also closely linked to the transport of cysteine across cell membranes (Hanigan & Rickets, 1993). It could be argued that inhibition of the glutamate transporter by L-αAA might produce a secondary effect on cystine transport, because these transport systems must inevitably be interlinked. In this context, it is worth noting that one mechanism of glutamate toxicity which operates via a disruption of cystine/cysteine transport is probably only apparent in young animals in which the glutamate transporter is not yet fully developed (Coyle & Puttfarcken, 1993).

In conclusion, these results confirm the stereospecificity of the glial cell toxicity of αAA, as it is the L-isomer that is the more effective inhibitor of both the transport of glutamate and of glutamine synthetase and γ-glutamylcysteine synthetase. Furthermore, the preference of L-αAA for glial cells is best explained by the relatively higher incidence of the transporter on these cells, compared to neurones (Drejer *et al.*, 1982) and also because glutamine synthetase is exclusively localized to glial cells, although the most relevant effect of L-αAA toxicity in glial cells is probably inhibition of the plasma membrane glutamate transporter. This study also highlights the importance of glial cells in maintaining the integrity of the surrounding neurones, and indicates that a disruption of glutamate metabolism in these cells, as opposed to neurones, is sufficient to cause a toxic reaction in the CNS. Further work will be aimed at analysing the impact of toxins like L-αAA on the free-radical detoxification mechanisms in the brain, and of how closely glutamate metabolism is linked to the production of anti-oxidants, such as glutathione.

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