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Post-ischaemic Alteration of Excitatory Amino Acid Transport Sites in the Gerbil Hippocampus

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Abstract

Sodium-dependent [^{3}H]D-aspartate binding as a marker of excitatory amino acid transport sites in the gerbil hippocampus was evaluated by quantitative receptor autoradiography 1 h to 7 days after transient cerebral ischaemia for 10 min.

Sodium-dependent [^{3}H]D-aspartate binding in the hippocampal CA1 and CA3 sectors significantly increased in the early post-ischaemic stage. After 7 days, a conspicuous elevation of sodium-dependent [^{3}H]D-aspartate-binding was observed in the hippocampal CA1 sector and dentate gyrus. However, no significant change in the binding was found in the hippocampal CA3 sector. A histological study revealed that transient ischaemia caused severe neuronal damage in the hippocampal CA1 sector and mild damage in the hippocampal CA3 sector. However, no ischaemic neuronal damage was observed in the dentate gyrus. An immunohistochemical study also showed that numerous reactive astrocytes were evident in the hippocampus, particularly in the hippocampal CA1 sector, 7 days after ischaemia.

These results demonstrate that transient cerebral ischaemia can cause marked elevation in excitatory amino-acid transport sites in the hippocampus. Furthermore, our results suggest that the post-ischaemic increase in excitatory amino acid transport sites might reflect expression of reactive astrocytes. These findings are of interest in relation to the mechanisms of ischaemic hippocampal damage.

It is well established that transient cerebral ischaemia leads to selective death of neurons in specific brain areas. The hippocampal CA1 pyramidal neurons are particularly vulnerable to ischaemia (Kirino 1982). Several microdialysis studies have demonstrated that extracellular levels of the excitatory amino acids, e.g. glutamate and aspartate, increase dramatically in the vulnerable regions after cerebral ischaemia (Benveniste et al 1984; Hagberg et al 1985). Furthermore, a previous study has suggested that the activation of glutamate receptor is involved in excitotoxic ischaemic neuronal damage (Rothman & Olney 1987). As a result of these observations, much interest is currently focused on the role of the excitatory amino acid glutamate in the development of ischaemic neuronal damage. However, specific glutamate antagonists have not been consistently protective in models of transient cerebral ischaemia (Albers et al 1989; Buchan & Pulsinelli 1990). Therefore, the detailed relationship between glutamate and ischaemic neuronal damage is still enigmatic.

The specific uptake system for glutamate and aspartate is the most important mechanism for regulation the synaptic activity of these excitatory amino acid neurotransmitters. Glutamate is rapidly removed from the synaptic cleft by re-uptake into glutamatergic nerve terminals and glia cells (Fonnum 1984). In particular, the sodium-dependent excitatory amino acid transport system plays a major role in the clearance of excitatory amino acids and so dysfunction of this transport system might contribute to extracellular overflow of these amino acids. Although these observations seem to suggest that the ability of the transport system to maintain extracellular levels of excitatory amino acids might play an important role in the pathogenesis of ischaemic neuronal damage, few investigations have been performed on the effect of ischaemia on the excitatory amino acid transport sites. In this study, therefore, we investigated sequential changes in excitatory amino acid transport sites in selectively vulnerable hippocampus after transient cerebral ischaemia in gerbils.

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Materials and Methods

Animals

Experiments were performed on male adult Mongolian gerbils, 60 to 80 g. The animals were anaesthetized with 2% halothane in a mixture of 30% oxygen and 70% nitrous oxide. Bilateral common carotid arteries were gently exposed and the arteries were occluded with aneurysm clips for 10 min; the gerbils were then allowed to survive for 1, 5, 24 and 48 h and 7 days after this transient ischaemia. Sham-operated animals were treated in the same manner, except for the clipping of the bilateral common carotid arteries. Body temperature was maintained at 37–38°C with a heating pad equipped with a thermostat until the animals began to move. For receptor autoradiographic study the animals were lightly anaesthetized with ether and then killed by decapitation. The brains were removed quickly and frozen in powdered dry-ice; coronal sections (12 μm) were cut on a cryostat at -20°C and thaw-mounted on to gelatin-coated slides. The sections were stored at -80°C until assay. For immunohistochemical study the animals were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.) 7 days after ischaemia, and the brains were perfusion-fixed with 4% paraformaldehyde in 0·1 M phosphate buffer (pH 7·4) after a heparinized saline flush. The brains were removed 1 h after perfusion-fixation and post-fixed for

4 h in the same fixative. The brains were immersed in sucrose solutions of graded strength (10 and 20%) in 0·1 M phosphate buffer over 2 days at 4°C and then frozen in powered dry-ice. Coronal sections (20 μm) were prepared on a cryostat at −20°C and thaw-mounted on to gelatin-coated slides. The frozen sections were stained with cresyl violet, and the sections were examined by light microscopy.

Receptor autoradiography
Autoradiographic localization of sodium-dependent [^{3}H]D-aspartate binding was performed by the method of Anderson et al (1991, 1993) with minor modifications. Brain sections were pre-incubated for 10 min at 30°C in 50 mM Tris-HCl buffer (pH 7·4). The sections were then incubated with 100 nM [^{3}H]D-aspartate (specific activity, 12·8 Ci mmol^{-1}) in 50 mM Tris-HCl buffer (pH 7·4) including 300 mM NaCl for 10 min at 2°C. After incubation the sections were washed four times in fresh, ice-cold buffer for a total of 30 s. Non-specific binding was determined by use of 100 μM DL-*threo*-β-hydroxy-aspartate (Sigma) under the same experimental conditions.

The sections were quickly dried under a stream of cold air and were exposed to Hyperfilm-^{3}H (Amersham) for 4 weeks in X-ray cassettes with a set of [^{3}H]microscales (Amersham). The optical density of the brain areas was measured with a computer-controlled image analyser, as described previously (Araki et al 1995). Binding assays were performed in duplicate under subdued lighting. Values were expressed as means ± s.d. Statistical significance was determined by analysis of variance then Dunnett's multiple comparison test. Each group contained five to eight gerbils.

Glial fibrillary acidic protein (GFAP) immunohistochemistry
A polyclonal anti-GFAP antibody (Labsystems, Oy, Helsinki, Finland) and a Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA) were used for immunohistochemical staining. The frozen sections were dried, washed for 5 min in 0·01 M phosphate-buffered saline (PBS, pH 7·4), then pre-incubated for 30 min with 10% normal horse serum. The brain sections were then incubated overnight at 4°C with anti-GFAP antibody (1:200). After a 10-min rinse in changes of PBS, the sections were incubated with biotinylated second antibody for 2 h and then with an avidin-biotin-peroxidase complex for 30 min at room temperature. Immunoreactions were visualized by use of diaminobenzidine (0·05%) and hydrogen peroxidase (0·01%) in Tris-HCl buffer (pH 7·6; 0·05%). Negative control sections were treated in the same way except that the antibody against anti-GFAP was omitted. Each group contained 5 animals.

Results

Receptor autoradiography
Autoradiograms of excitatory amino acid transport sites and histology are shown in Fig. 1. Post-ischaemic changes in sodium-dependent [^{3}H]D-aspartate binding are summarized in Table 1. For gerbils subjected to ischaemia a transient increase in sodium-dependent [^{3}H]D-aspartate binding in the hippocampal CA1 (stratum oriens, stratum radiatum and stratum lacunosum-moleculare) and CA3 (average) sectors was observed within the hippocampal formation 1 h after recirculation. Five hours after ischaemia a significant increase in

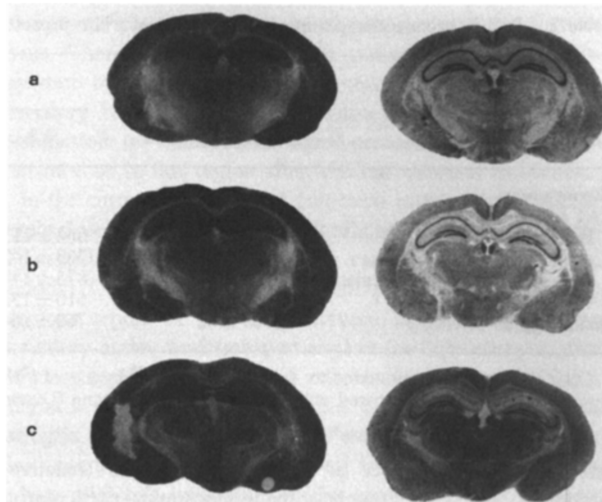

FIG. 1. Autoradiograms of sodium-dependent [^{3}H]D-aspartate binding and photographs with cresyl violet staining of the gerbil hippocampus after cerebral ischaemia. Left, sodium-dependent [^{3}H]D-aspartate binding; right, cresyl violet staining. a. sham-operated, b. 1 h after ischaemia, c. 7 days after ischaemia. A significant increase in sodium-dependent [^{3}H]D-aspartate binding was observed in the hippocampal CA1 and CA3 sectors in gerbils 1 h after ischaemia (b), compared with those in sham-operated animals (a). However, no conspicuous neuronal damage was observed in the hippocampus 1 h after ischaemia (a, b). Seven days after ischaemia a significant increase in sodium-dependent [^{3}H]D-aspartate binding was observed in the hippocampal CA1 sector and dentate gyrus (c). Furthermore, severe neuronal damage was noted in the hippocampal CA1 sector and mild damage was seen in the hippocampal CA3 sector. However, the dentate gyrus was intact (c).

sodium-dependent [^{3}H]D-aspartate binding was still evident in the stratum radiatum of the hippocampal CA1 and CA3 sectors. Thereafter, these regions showed no significant changes in sodium-dependent [^{3}H]D-aspartate binding 24 and 48 h after ischaemia. In contrast, no conspicuous changes in sodium-dependent [^{3}H]D-aspartate binding was apparent in the dentate gyrus up to 48 h after recirculation. After 7 days, a significant increase in sodium-dependent [^{3}H]D-aspartate binding was observed in the hippocampal CA1 sector and dentate gyrus.

Histology
As is apparent from Fig. 1, no neuronal damage was apparent throughout the hippocampus of sham-operated gerbils. No conspicuous neuronal damage was observed up to 48 h in the hippocampus of gerbils subjected to ischaemia. After 7 days the most frequently affected area was the hippocampal CA1 sector. The hippocampal CA3 sector was mildly damaged. However, the dentate gyrus was morphologically intact. These results are consistent with our previous report (Araki et al 1993).

GFAP immunohistochemistry
Representative microphotographs of GFAP immunostaining in the gerbil hippocampus are shown in Fig. 2. A ramified form of GFAP-positive astrocytes with many fine processes was observed in the hippocampus of sham-operated animals. However, the astrocytes were absent in the pyramidal cell and dentate granule cell layers. Seven days after ischaemia, GFAP immunoreactivity of individual astrocytes became more

Table 1. Post-ischaemic changes in sodium-dependent [^{3}H]D-aspartate binding in the gerbil hippocampus.

Region	Sham-operated	Recirculation time				
		1 h	5 h	24 h	48 h	7 days
Hippocampus						
CA1 sector						
stratum oriens	533 ± 52	662 ± 113*	622 ± 97	541 ± 70	499 ± 47	689 ± 65*
stratum radiatum	531 ± 60	769 ± 142†	696 ± 110*	577 ± 73	546 ± 75	724 ± 88*
stratum lacunosum-moleculare	653 ± 108	875 ± 148*	810 ± 124	663 ± 98	635 ± 102	1036 ± 237†
CA3 sector (average)	434 ± 52	610 ± 158*	538 ± 54*	435 ± 76	412 ± 60	443 ± 52
Dentate gyrus (average)	709 ± 82	768 ± 164	825 ± 43	697 ± 77	692 ± 106	913 ± 130*

Optical density was converted to fmol (mg tissue)$^{-1}$ by use of [^{3}H]microscales. Values are expressed as means ± s.d. *$P < 0.05$, † $P < 0.01$, significantly different compared with the sham-operated group (Dunnett's multiple range test; n = 5—8).

intense and the number of GFAP-positive cells (reactive astrocytes) increased throughout the hippocampus, particularly in the hippocampal CA1 sector where severe neuronal damage was noted.

Discussion

It is known that [^{3}H]D-aspartate as a ligand shows specific and high affinity for sodium-dependent transport and this compound has been used extensively to characterize transport and binding properties in brain tissue (Taxt & Storm-Mathisen 1984; Anderson et al 1990). Furthermore, quantitative autoradiography of sodium-dependent [^{3}H]D-aspartate binding in the brain of experimental animals has previously been described (Parsons & Rainbow 1983) and the technique has been applied to the study of post-mortem tissue from man (Charlton & Candy 1988; Slater et al 1992). Thus, [^{3}H]D-aspartate has been proposed as a suitable ligand for use in binding studies of excitatory amino acid transport sites, particularly glutamate transport sites, in the brains of man and experimental animals. Interestingly, Robinson et al (1993) demonstrated that L-*trans*-pyrrolidine-2,4-dicarboxylate, a selective inhibitor of the glutamate transport system, increased glutamate toxicity approximately fivefold in primary hippocampal cultures. Rothstein et al (1993) also showed that selective inhibition of glutamate transport with DL-*threo*-3-hydroxyaspartate continuously increased glutamate concentrations in the medium of cultured organotypic spinal cord slices. Furthermore, a previous study indicated that injection of DL-*threo*-3-hydroxyaspartate into the striatum resulted in neurodegeneration (McBean & Roberts 1985). These observations seem to suggest that the dysfunction of the excitatory amino acid transport system might increase the vulnerability of neurons to the toxic effects of synaptically released glutamate.

The current study showed that in selectively vulnerable regions, the hippocampal CA1 and CA3 sectors, sodium-dependent [^{3}H]D-aspartate binding was significantly elevated early in the recirculation period without neuronal cell loss. Thereafter, a significant elevation in sodium-dependent [^{3}H]D-aspartate binding in the hippocampal CA1 and CA3 sectors returned to sham-operated levels 24 and 48 h after ischaemia. In contrast, no significant change in sodium-dependent [^{3}H]D-

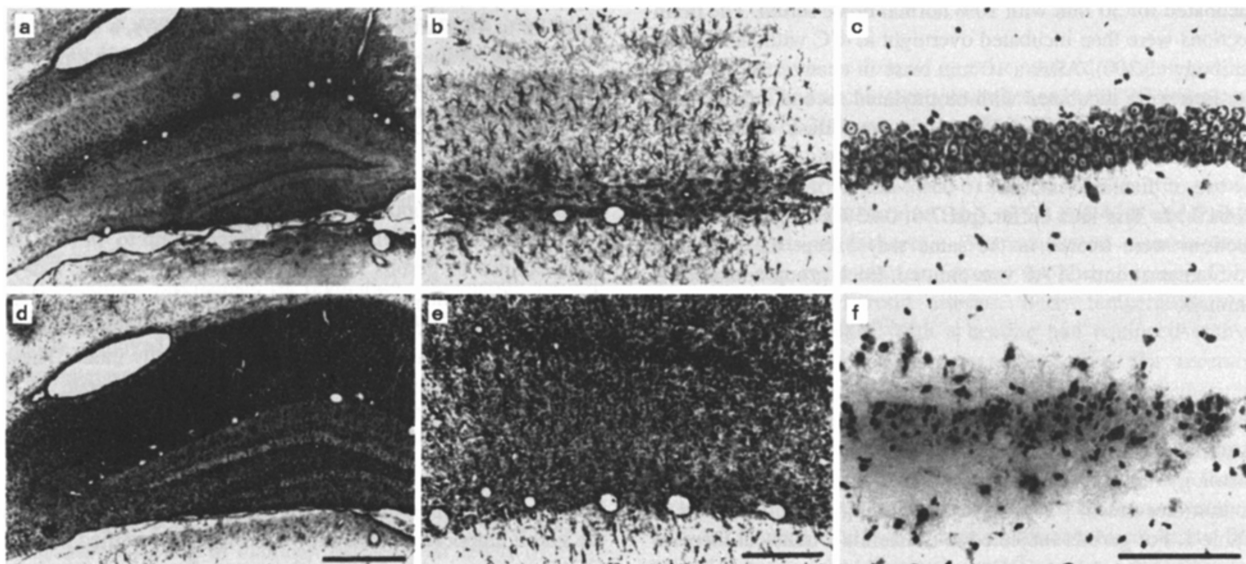

FIG. 2. GFAP (glial fibrillary acidic protein) immunostaining and histological findings with cresyl violet staining in the gerbil hippocampus after cerebral ischaemia. a-c. sham-operated, d-f. 7 days after ischaemia. GFAP-positive cells were particularly observed in the hippocampal CA1 sector (a, b). The pyramidal neurons of the hippocampal CA1 sector were morphologically intact (c). Numerous reactive astrocytes were observed in the hippocampal CA1 sector and dentate gyrus (d, e). The hippocampal neurons were completely destroyed and numerous glia cells were observed in hippocampal CA1 sector (f). Bar = 500 μm (a, d); 200 μm (b, e); 100 μm (c, f).

aspartate binding was observed up to 48 h after recirculation in the dentate gyrus, a region particularly resistant to ischaemia. Seven days after ischaemia, however, marked elevation in sodium-dependent [^{3}H]D-aspartate binding was found in the hippocampal CA1 sector and dentate gyrus. No significant change in sodium-dependent [^{3}H]D-aspartate binding was observed in the hippocampal CA3 sector. These results demonstrate that transient cerebral ischaemia can cause a significant elevation in sodium-dependent [^{3}H]D-aspartate binding in the hippocampal CA1 and CA3 sectors early in the recirculation period. Furthermore, our results suggest that this increase in sodium-dependent [^{3}H]D-aspartate binding precedes ischaemic neuronal damage in the hippocampal CA1 and CA3 sectors.

In the current study, however, no significant change in sodium-dependent [^{3}H]D-aspartate binding was found in the hippocampal CA3 sector 7 days after ischaemia, whereas a significant increase in the binding was observed at the same time-point in the hippocampal CA1 sector. Furthermore, a significant increase in sodium-dependent [^{3}H]D-aspartate binding was observed in the dentate gyrus 7 days after ischaemia. Thus, the increase in sodium-dependent [^{3}H]D-aspartate binding in the hippocampal CA1 sector and dentate gyrus was observed 7 days after ischaemia, despite the loss of hippocampal neurons. This finding suggests that sodium-dependent [^{3}H]D-aspartate binding might be predominantly located on non-neuronal elements. Interestingly, a previous study suggested that the increase in sodium-dependent [^{3}H]D-aspartate binding in the hippocampus after an entorhinal lesion paralleled the appearance of reactive astrocytes as assessed with GFAP immunohistochemistry (Anderson et al 1991). Lin et al (1993) also reported that transient cerebral ischaemia induced an increase in GFAP immunoreactivity which can be detected in the hippocampus and which became prominent by 4–7 days. Our immunohistochemical study also showed that numerous reactive astrocytes were evident in the hippocampus, particularly in the hippocampal CA1 sector, 7 days after ischaemia. Therefore, the current study provides evidence that the increase in sodium-dependent [^{3}H]D-aspartate binding in the hippocampal CA1 sector might reflect the expression of reactive astrocytes after transient cerebral ischaemia. On the other hand, we could not detect an increase in sodium-dependent [^{3}H]D-aspartate binding in the hippocampal CA3 sector where neuronal damage was seen. The precise reason for this phenomenon is presently unclear, although it might be explained by the evidence that no marked increase in reactive astrocytes was observed in the hippocampal CA3 sector, because ischaemic neuronal damage in this region was milder than that in the hippocampal CA1 sector. In contrast, a significant elevation in sodium-dependent [^{3}H]D-aspartate binding was observed in the dentate gyrus, which was resistant to ischaemia, only 7 days after recirculation. We previously reported that transient cere-bral ischaemia in gerbils can induce a significant increase in [^{3}H]phorbol-12,13-dibutyrate and a significant reduction in [^{3}H]inositol-1,4,5-trisphospate and [^{3}H]glycine-binding in the dentate gyrus 7 days after recirculation (Araki et al 1992, 1995). We also observed that microtubule-associated protein 2 immunoreactivity increased in the molecular layer of dentate gyrus 7 days after ischaemia (Araki et al 1995). From these observations, our present findings seem to suggest post-ischaemic dysfunction of intracellular signal transduction and neurotransmission in the dentate gyrus. Therefore, this increase in sodium-dependent [^{3}H]D-aspartate binding in the dentate gyrus might reflect a compensatory response of reactive astrocytes produced by the dysfunction of intracellular signal transduction and neurotransmission in this region after transient cerebral ischaemia.

In the current study it is of particular interest that transient cerebral ischaemia can cause a significant increase in sodium-dependent [^{3}H]D-aspartate binding early in the recirculation periods. This finding is, at least in part, consistent with a previous report of the early increase in sodium-dependent excitatory amino acid transport sites in the hippocampus after transient cerebral ischaemia in rats (Anderson et al 1993). A previous study demonstrated rapid biochemical changes in astroglia after ischaemia in rats (Petito & Babiak 1982). We also reported that transient cerebral ischaemia in gerbils can induce glial cell infiltration in the hippocampus at an early stage after recirculation (Araki et al 1994). Casado et al (1991) reported that phorbol ester, a potent activator of protein kinase C, can stimulate sodium-dependent glial glutamate uptake, indicating that protein kinase C might regulate glutamate transport systems. Furthermore, it is well known that protein kinase C levels increase markedly during ischaemia (Cardell & Wieloch 1993). From these observations, it is conceivable that the early increase in sodium-dependent [^{3}H]D-aspartate binding in the current study might be a consequence of the compensatory reaction of astroglia to protect neurons from ischemic insult. However, sodium-dependent [^{3}H]D-aspartate binding is known to be localized on neurons (Fonnum 1984). Therefore, further studies are needed to clarify our findings in detail.

In conclusion, this study provides evidence that transient cerebral ischaemia causes marked elevation in excitatory amino acid transport sites in the hippocampus. Our results also demonstrate that the post-ischaemic increase in excitatory amino acid transport sites might reflect expression of reactive astrocytes.

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