NITROPRUSSIDE INHIBITS KINDLING DEVELOPMENT AND POTENTIATES EXCITATORY AMINO ACID EXOCYTOSIS IN THE KINDLING RAT

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Abstract

The effect of sodium nitroprusside (SNP), one of the nitric oxide (NO) donors, on the development of acute amygdaloid kindling and amino acid exocytosis in the hippocampus of kindled rats was examined using *in vivo* microdialysis. The number of stimulations required for rats perfused with 10 mM SNP through the ventral hippocampus prior to the first kindling stimulation was larger than that of control, and the afterdischarge duration was shorter than that of control. On the other hand, SNP-induced enhancement of aspartate, glutamate and glycine overflow in the established kindled rats was greater than that in control. This SNP-induced enhancement of amino acid overflow was TTX-insensitive. These results suggest that NO has at least two roles in kindling; to accelerate amino acid exocytosis, and to inhibit kindling development.

Introduction

Kindling is a widely accepted model of secondary generalized seizure in which repeated subconvulsive electrical stimulation results in progressively more intense seizures. Once kindled, increased sensitivity to subconvulsive stimulation is persistent (Goddard *et al.*, 1969; Maru and Goddard, 1987). A condition in which initially subconvulsive stimulus elicits augmented seizures is an experimental analogue of complex partial epilepsy. Although well characterized phenomenologically, the basic mechanism responsible for the development of kindling is unknown.

Recent reports have explored the possibility that long-lasting excitatory synaptic transmission efficacy in the kindling model is closely related to extracellular glutamate (Glu) concentration and Glu-mediated responses of N-methyl-D-aspartate (NMDA) receptors, combined with the collapse of γ-aminobutyric acid (GABA)-mediated inhibition (Kapur and Lothman,1990; Morimoto, 1989). It has been already reported that

the repetitive increases in extracellular Glu and GABA concentration after each kindling stimulation are closely accompanied by an imbalance between excitatory and inhibitory system (Ueda and Tsuru, 1995).

In kindling development, it not only is the imbalance between the excitatory and inhibitory system important, but also the participation of free radicals, including nitric oxide (NO). It has already been reported that inhibitors of NO synthase (NOS) increase kindling rate (Rondouin *et al.*, 1992), and that NO itself modifies the overflow of neurotransmitters, such as aspartate (Asp), Glu and dopamine (Lonart *et al.*, 1992; Zhu and Luo, 1992).

There are very few reports on the direct effect of NO on kindling development or on the modification of neurotransmitter exocytosis in the fully kindled state. The aim in this experiment was to examine the effect of sodium nitroprusside (SNP: donor of NO) perfusion in the ventral hippocampus on the development of acute amygdaloid kindling, and the sequential change in the extracellular level of amino acids before, during and after perfusion of SNP, in fully kindled rats using *in vivo* brain microdialysis.

Materials and Methods

Thirty seven male Wistar rats weighing 380-420 g at the time of surgery were anesthetized with pentobarbital sodium (37.5 mg/kg, i.p.). A single tripolar stainless steel electrode (0.2 mm diameter), for stimulation and electroencephalogram (EEG) recording, was implanted into the basonucleus of the right amygdaloid body. In this experiment, stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1986). The incisor bar was set 3.3 mm below the intra-aural line. Stereotaxic coordinates for the electrodes were: 6.0 mm anterior and 5.0 mm right of the lambda, and 8.5 mm below the surface of the skull.

At the time of electrode implantation, each rat was also stereotaxically implanted with a 22-G guide cannula coupled with a dummy cannula. Stereotaxic coordinates were: 5.6 mm posterior, 5.0 mm left of the bregma, and 3.0 mm below the surface of the skull. The electrode plug and 22-G guide cannula with dummy cannula were firmly anchored to the skull with miniature screws and dental cement.

Seven days after the implantation of the electrode and guide cannula, the rats were divided into 5 (I-A, I-B, II-A, II-B, II-C) groups. Two types of experiments were conducted.

Experiment I

To investigate the effect of SNP on kindling development, the rats from two groups, I-A (n = 8) and I-B (n = 8), were administered different types of solution to the ventral hippocampus prior to the first stimulation of acute kindling. The administration of solution to the ventral hippocampus was carried out using perfusion, by *in vivo* brain microdialysis. For microdialysis, the dummy cannula was replaced with a dialysis cannula consisting of a 24-G introducer needle, of which the tip was covered with a 4 mm length of permeable hollow fiber (11 μm thickness, 0.2 mm outside diameter, molecular cut-off 7,000-8,000, Cuprophan, Nikkiso, Japan). The dialysis cannula was connected to a microinfusion pump (BRC, Japan) and continuously perfused with Ringer solution (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$: pH 6.5) at 2.0 μ/min.

Each rat of group I-A was perfused for 20 min with vehicle (Ringer solution) in the ventral hippocampus, while each rat of group I-B was perfused for 20 min with 10 mM SNP resolved in Ringer solution. After perfusion, acute kindling was induced in each rat. Electrical subconvulsive stimulations (200 μA) were given to the basolateral nucleus of the right amygdaloid body. The stimulus consisted of a 2 sec train of 60 Hz biphasic square wave current of 1 msec duration. Stimuli were delivered every one hour (8-9 stimuli/day) until each rat was fully kindled. At the time of stimulation, after-discharge duration (ADD) was measured and the severity of the seizure was rated by Racine's scale (Racine, 1972).

Experiment II

In this experiment, the amino acid overflow affected by SNP perfusion was examined in the control (II-A) and the fully kindled group (II-B). Moreover, whether the NO-induced changes of amino acid exocytosis are TTX-sensitive or not was also investigated using group II-C.

For group II-B (N = 8), acute kindling was induced in the same way as in EXP. I. Two hours after the last kindled seizure, *in vivo* microdialysis was performed as mentioned in EXP. I. After a 2 hr stabilization period, dialysates were collected every 20 min (=40 μl of dialysate) for 140 mins. *In vivo* microdialysis was performed on each of the rats from group II-A and II-C, using the same procedure as that for group II-B. Among the 7 successive dialysates, the 3rd was the perfusion of 10 mM SNP in group II-A and II-B, and 10 mM SNP + 1 μM TTX in group II-C. Extracellular amino acid concentration prior to the perfusion of SNP was used as the baseline concentration in each group.

Quantitative Analysis of Amino Acids

The following amino acids were detected: Asp, Glu, glutamine (Gln), glycine (Gly), taurine (Tau), alanine (Ala), and GABA. Immediately after collection, all sampling tubes were stored at -70°C. Amino acid determination was carried out using high performance liquid chromatography with electrochemical detection (HPLC-ECD). Before subjecting the dialyzates to HPLC-ECD, *o*-phthalaldehyde (OPA) solution was made by adding 13.5 mg OPA and 10 μl 2-mercaptoethanol, to 2.5 ml of 0.1 M K_2CO_3 buffer (pH 9.6) with 10 % ethanol. The samples (40 μl of dialyzate) were mixed with 10 μl OPA solution and allowed to react for 2.5 min at 25°C. After completion of the reaction, 15 μl was applied to an ODS column for HPLC. Detection was done by ECD (Eicom Co. Ltd, Tokyo, Japan) with + 650 mV/Ag/AgCl. The elution buffer was 0.05 M NaH_2PO2H_2O, 50% methanol, and 0.5 mM EDTA (pH 3.5). Amino acid concentrations in brain dialysates were calculated from the known amounts (0.2 μM/40 μl).

Following the experiment, each rat was given an overdose of pentobarbital and perfused transcardially with 10 % formalin. The brain was then removed, sectioned to thicknesses of 50 μm, and stained with neutral red. The position of the dialytrodes and the area of the stimulatory electrode were examined histologically.

Statistical Analysis

Results were analyzed as follows: statistical comparisons for the effect of 10 mM

SNP perfusion on kindling development between groups I-A and I-B, and for the sequential changes in extracellular amino acid concentration among groups II-A, II-B, and II-C, were carried out using the 2-way ANOVA followed by the Tukey test for multiple comparison. The baseline concentrations of amino acids of II-A were compared with those of groups II-B and II-C using the Mann-Whitney U-test.

Results

Histological Examination

The point at which the guide cannula had touched the surface of the hippocampus, and the dialysis membranes 4 mm in length, were located in the ventral hippocampi in each experimental animal.

TABLE I

The baseline concentration of amino acids in group II-A, II-B and II-C (pmol/40 μl dialysate sample)

	II-A	II-B	II-C
Asp	3.35 ± 1.27	7.9 ± 1.27*	3.11 ± 2.11
Glu	11.11 ± 3.35	37.01 ± 13.99*	9.44 ± 6.44
Gln	79.76 ± 24.05	97.57 ± 36.88*	80.441 ± 6.44
Gly	16.51 ± 4.97	33.92 ± 12.82*	16.44 ± 5.69
Tau	7.48 ± 2.26	17.05 ± 6.44**	8.64 ± 1.98
Ala	12.83 ± 3.87	31.49 ± 11.90**	11.04 ± 3.14
GABA	0.70 ± 0.21	0.63 ± 0.24	0.58 ± 0.15

*$p < 0.05$, ** $p < 0.01$ vs. group II-A (Mann-Whitney U-test)

Effect of 10 mM SNP Dialysis on the Kindling Development and ADD:

A greater number of stimulations was needed to establish fully kindled seizure in group I-B rats than in group I-A. After-discharge duration (ADD) was shorter for group I-B than for group I-A (Figure 1).

Baseline Concentrations of Amino Acids:

Table 1 shows the baseline concentrations of amino acids in the corresponding groups. Glu, Gln, Gly, Tau and Ala concentrations in group II-B (the fully kindled

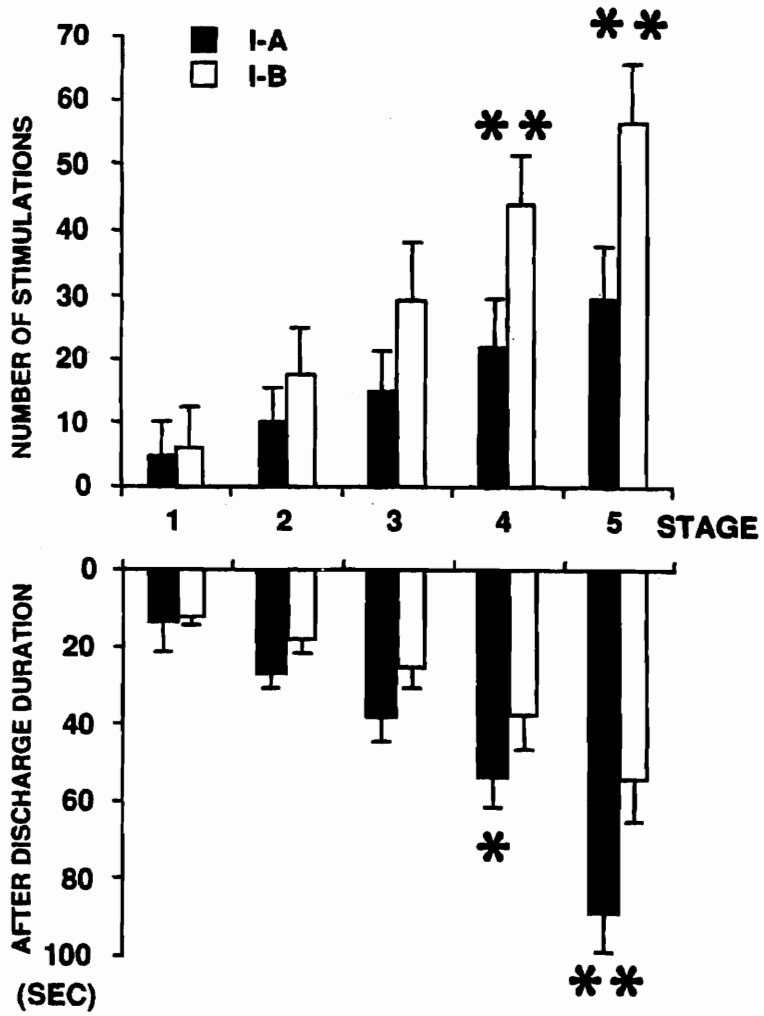

Figure 1. A greater number of stimulations was needed to establish fully kindled seizure activity in group I-B rats than in group I-A rats. Group I-B rats were perfused with 10 mM SNP through the ventral pocampus (upper graph). After-discharge duration (ADD) was shorter in group I-B than in group I-A, at the corresponding stage of kindling (lower graph). *$p < 0.05$, **$p < 0.01$, compared to the corresponding value of group I-A. (F-values for Group I-A and I-B effect of stimulations; $F_{1,56} = 101.224$, $p = 0.0001$; ADD, $F_{1,56} = 25.884$, $p = 0.0002$).

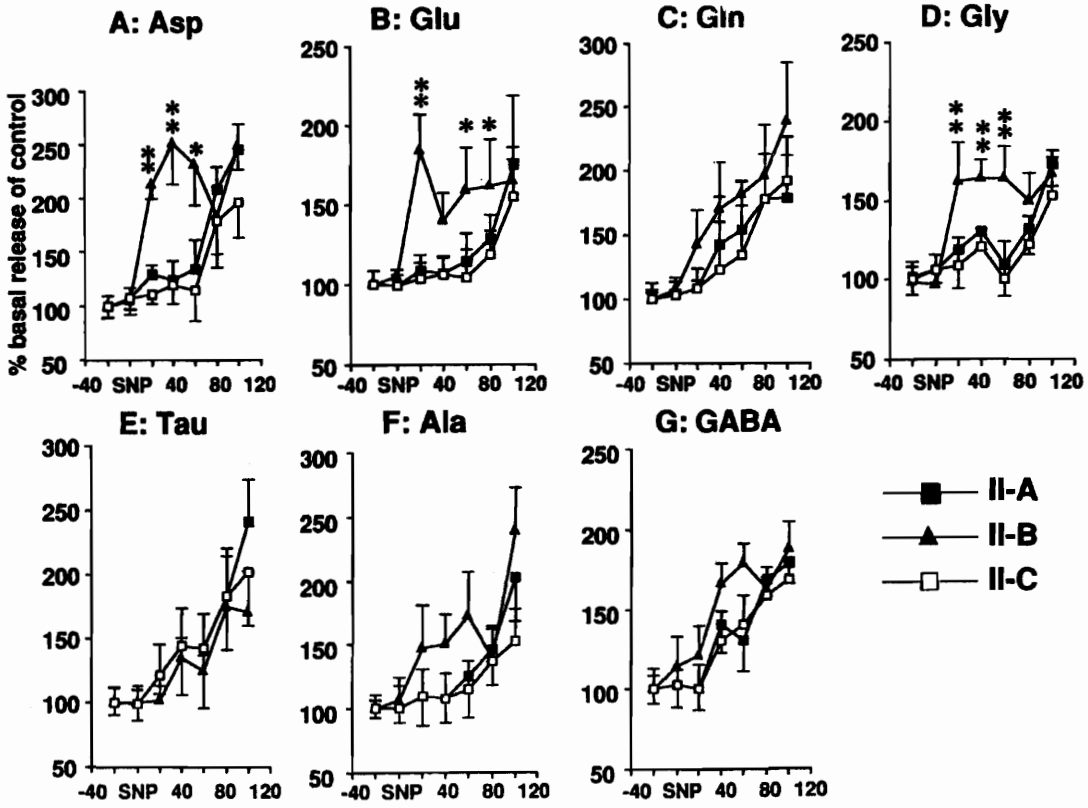

Figure 2. Sequential changes in extracellular concentrations of amino acid in the ventral hippocampal dialyzates from 40 min prior to 100 min after SNP perfusion. The values are means ± S.E.M. calculated as a percent change from the baseline concentrations shown in Table I. *$p < 0.05$, **$p < 0.01$, compared to the corresponding value of group II-A. (F-values for Group [II-A, B and C] effect of Asp; $F_{2,126} = 16.7$, $p = 0.0001$; Glu, $F_{2,126} = 24.6$, $p = 0.0001$; Gly, $F_{2,126} = 19.8$, $p = 0.0001$.) There were no significant differences in exocytosis of any amino acid between group II-A and II-C.

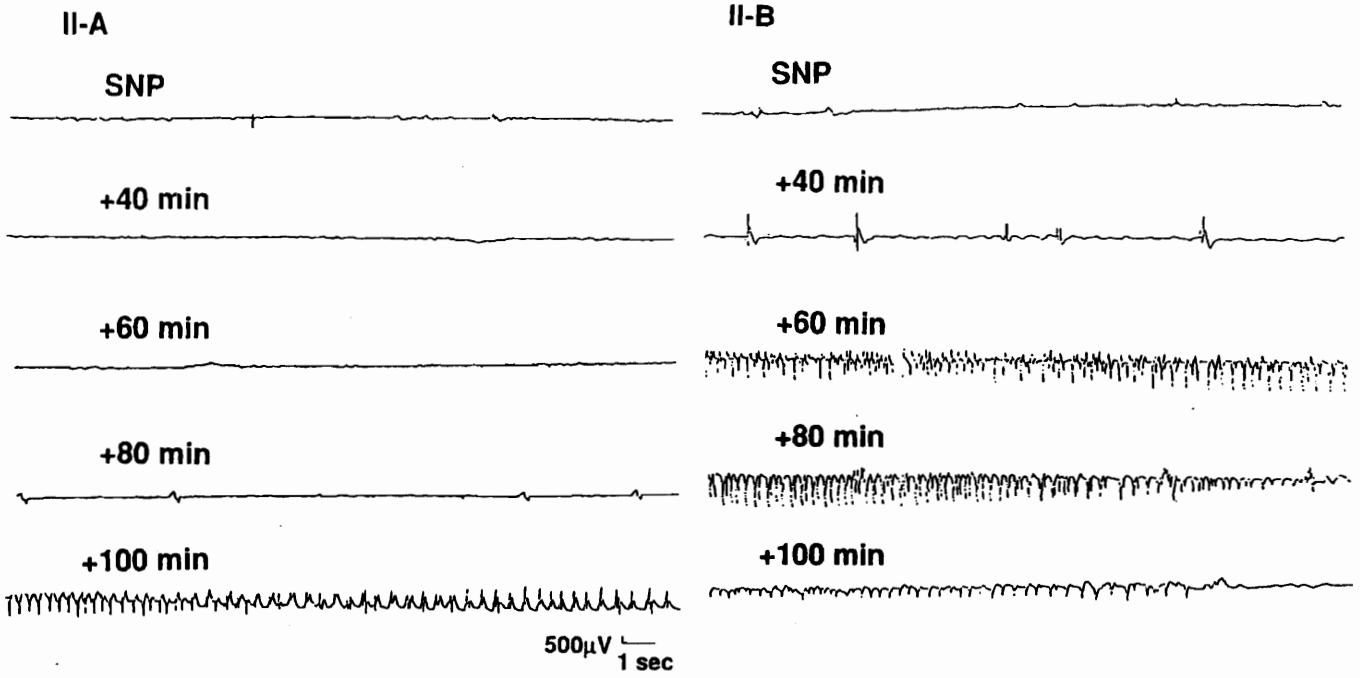

Figure 3. All the rats in group II-A were found to exhibit wet-dog shaking and have epileptiform discharge based on EEG recordings from the right amygdaloid body, about 100 min after the perfusion of 10 mM SNP. In group II-B, wet-dog shaking and epileptiform discharge began earlier, at about 40 min after the perfusion of SNP.

group) were enhanced hr after the fully kindled seizure, as compared to groups II-A and II-C.

Effect of the Perfusion of 10 mM SNP on the Sequential Changes in Extracellular Amino Acid Concentration in the Ventral Hippocampus

The values in Figure 2 are means ± S.E.M. calculated as a percent change from the baseline shown in Table I. Exocytosis of all amino acids was increased after the third collection; SNP perfusion through the hippocampus in each group. The increasing ratio of amino acid overflow induced by NO in group II-B was greater than that of group II-A and II-C. The remarkable increases in Asp, Glu and Gly concentrations after the perfusion of SNP in group II-B rats are significant compared to group II-A and II-C (Figure 2).

The Behavioral and the Electroencepharographic (EEG) Activity after the Perfusion of SNP in the Ventral Hippocampus:

All rats in group II-A and II-C were found to exhibit wet-dog shaking and have epileptiform discharge, based on EEG recordings from the right amygdaloid body, about 100 min after the perfusion of 10 mM SNP. In group II-B, wet-dog shaking with epileptiform discharge began earlier, at about 40 min after the perfusion of SNP, as shown in Figure 3.

Effect of 1 μM TTX on 10 mM SNP-related Changes in Extracellular Amino Acid Concentration:

Ten mM SNP-related changes in the concentrations of extracellular amino acids were not affected by adding 1 μM TTX to the perfusion (see Figure 2).

Discussion

The kindling phenomenon, shares some, but not all, of molecular mechanisms associated with LTP. Many investigators have demonstrated a particular role of NO, synthesized after NMDA receptor activation, and concluded that induction and

maintenance of LTP involve essentially presynaptic mechanisms, in particular, an increase in cGMP levels in response to the retrograde effect of NO.

Kindling, as a model of neuronal plasticity, results in progressively more intense seizures by repeated subconvulsive stimulation. Investigation of NO-induced changes in extracellular amino acid concentrations, especially Asp, Glu and GABA, in the ventral hippocampus, which is thought to be a crucial region for the development of kindling (Minamoto *et al.*, 1992), is necessary to understand the progressive enhancement of epileptogenecity *in vivo*, and to clarify the mechanism of excessive propagation of seizure activity found in the kindling model.

Experiment I clearly showes that NO itself applied prior to the first stimulation through the hippocampus inhibited acute amygdala-kindling development. We observed a slower progression of electrical as well as behavioral components during development of kindling in the rats of group I-B, which were perfused with the NO donor through the ventral hippocampus at the early phase of the procedure. Kindling development was suppressed, and ADD at the corresponding stage was shorter in group II-B than in group I-A. The number of stimulations required to reach stage 4-5 was significantly larger in group I-B than in group I-A. If kindling depends on presynaptic events which involve an increase in cGMP levels, we should find an accelerated ratio in kindling development and progression of ADD in group I-A. This was not the case.

However, the inhibitory effect of SNP on kindling development could also be due to a postsynaptic NO effect. Indeed, recent reports (Hoyt *et al.*, 1992; Lipton *et al.*, 1993) demonstrated that a novel feedback inhibitory mechanism by NO, inhibited NMDA receptor activation, which is thought to be important at the early phase of kindling in vitro (Miller, 1991; Rainnie *et al.*, 1992). Therefore, NO, generated by perfusion of SNP in the early phase of kindling procedure, may exert a negative feedback effect on NMDA receptor activity and lead to suppression of kindling development and the progression of ADD (see Figure 1).

Results of Experiment II show that NO from 10 mM SNP promptly increased the extracellular concentrations of amino acids in all groups. The basal release of Asp and Glu in group II-B, the kindled group, was remarkably higher than that in group II-A. Our

results are consistent with those of a previous report in which the basal release of Asp and Glu in kindled rats was higher than that in control rats, using *in vivo* microdialysis (Minamoto *et al.*, 1992). TTX did not affect neurotransmitter exocytosis enough to conclude that overflow was due to non-synaptic events, which means that exocytosis of these amino acid by SNP is due to leakage from neuronal cell bodies and/or glia. However, the sequential changes in the extracellular concentrations of amino acids affected by 10 mM SNP perfusion in kindled rats (group II-B) was quite different from those of controlled rats (group II-A). This difference probably contributed to some alteration in signal transduction, possibly at the level of the second messenger system in the brain. In addition, NO-induced exocytosis of excitatory amino acids(EAAs), that is Asp and Glu, would cause the early expression of epileptiform discharge and behavior observed in group II-B rats. In the kindled group, the increase in Asp overflow was about 230% of basal release, while that of Glu was about 200%. These NO-mediated increases were statistically larger than those of group II-A. This suggests that NO might induce an increase in Asp and Glu overflow in the extracellular space, at the final stage of kindling (stage 4), and that these EAAs might activate non-NMDA receptors, such as AMPA-sensitive, kainate-sensitive and metabotropic receptors (Miller, 1991; Rainnie *et al.*, 1992). These receptors are thought to be more closely related to the induction and the maintenance of convulsion than NMDA receptors in the kindling model (Miller, 1991; Rainnie *et al.*, 1992).

The results of ExperimentI and II suggest that NO has at least two roles in the development of kindling: to accelerate the exocytosis of amino acids, and to inhibit epileptogenesis. In conclusion, endogenous NO generated following Glu-mediated activation of NMDA receptors, might suppress the NMDA receptor at the early phase, and gradually activate non-NMDA receptors following kindling, as a result of exocytosis of EAAs in the extracellular space in the brain.

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