



Effect of kindled seizures on rat behavior in water Morris maze test and amino acid concentrations in brain structures

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Abstract:

The effects of kindled seizures elicited by repeated pentetrazole (PTZ) injections, on learning and memory in the Morris water maze test and on concentration of brain amino acids, were examined in rats. It was found that kindled seizures (a model of temporal lobe epilepsy) produced a profound decrease in learning and memory accompanied by a selective and long-lasting decrease in hippocampal and striatal concentration of glutamate, glycine and alanine in the striatum (*ex vivo* measurement). The concentrations of histamine, serine and γ -aminobutyric acid (GABA) were not selectively affected by kindling. A lower concentration of glutamate and N-methyl-D-aspartate (NMDA) receptor co-agonists in the striatum (glycine and alanine) indicates the general malfunction of the brain glutamatergic system. It is suggested that a selective decrease in hippocampal glutamate concentration may account for deterioration in learning and memory processes in kindled rats, considering the important role of this neurotransmitter in the cognitive processes (e.g. in the long-term potentiation), and the key contribution of the hippocampus to the spatial memory. The intrinsic mechanisms of the reported behavioral effects may involve neuronal damage in the brain limbic structures, secondary to seizure-induced ischemia and hypoxia.

Key words:

pentetrazole (PTZ), kindled seizures, Morris water maze, glutamate, hippocampus

Introduction

Kindled seizures are widely accepted as an animal model of temporal lobe epilepsy (TLE), wherein repeated subthreshold brain stimulation (electrical or chemical) leads to behavioral signs of seizures (tonic and clonic) [11, 19], and such procedure results in a state mimicking many symptoms of human TLE:

hippocampal atrophy, loss of neurons, gliosis and growth of new neuronal connections [22, 31, 32].

Kindled seizures cause a neuronal loss also in the limbic areas: CA1, CA3, dentate gyrus of the hippocampus, amygdala, and entorhinal cortex [6, 24]. The neuronal damage in the hippocampus is related to the accompanying memory dysfunction, since the hippocampus is well recognized as a structure participating in memory processes (spatial and contextual

memory), and impairment to this brain area can produce severe amnesia. Spatial-memory deficits have been observed in many species (including rodents and humans) with hippocampal damage [3, 9, 15, 26, 28]. Disturbances of memory may be the consequence of seizure-induced biochemical changes in the brain, related to glutamate and aspartate release [10, 12, 18].

It is well recognized that seizures are the outcome of an imbalance between central excitatory and inhibitory processes. Research on the mechanisms of epilepsy have focused on changes in the activity of the inhibitory (GABA), and the excitatory amino acids (glutamate and aspartate). In some studies, an elevation of glutamate levels in the hippocampus during, or immediately after fits of seizures was found in humans as well as in rats [5, 20]. Similar effects on GABA concentration have also been shown [34]. Much less is known about the effects of seizures on brain concentrations of other amino acids. Alterations in the function of several amino acids have been reported in the genetically epilepsy-prone rats as well as in the pentetrazole model of epilepsy [17, 30]. Very little is known about changes in the activity of amino acids in a silent period, between fits of seizures.

Bearing in mind the fact that learning and memory can be influenced by the process of epileptogenesis, and by the changes in the activity of amino acids, we have decided to further investigate the relationship between amino acid concentrations and the behavior of kindled animals (PTZ) in the Morris water maze. Alterations in local contents of amino acids were studied in the hippocampus, striatum and prefrontal cortex, given their contribution to the cognitive and motor aspects of seizures. Additionally, the bioavailability of pentetrazole (PTZ) was studied, to assess whether individual variability of rats' response to PTZ-induced kindling, and changes in local concentrations of amino acids were not due to the differences in its penetration to the brain.

Materials and Methods

Animals

Adult male Wistar rats, bought from a licensed breeder, weighting 200 ± 20 g at the beginning of the experiment were used in the study. The animals were

housed two per cage in standard laboratory conditions under 12 h light-dark cycle (lights on at 6 a.m.) at controlled temperature ($20 \pm 2^\circ\text{C}$) and 70% humidity. The rats were given free access to food and water. All experiments were approved by the Committee for Animal Care and Use at the Medical University in Warszawa. The study was carried out in accordance with European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs and treatments

PTZ was dissolved in saline and injected intraperitoneally in a volume of 1 ml/kg, at a subconvulsive dose of 35 mg/kg three times a week (Monday, Wednesday and Friday). After each injection, the rats were placed singly in isolated transparent Plexiglas cages and were observed for 30 min. The intensity of convulsions was rated according to a 5-point scale: 0 – no response, 1 – ear and facial twitching, 2 – myoclonic jerks without rearing, 3 – myoclonic jerks, rearing, 4 – turn over into side position, clonic – tonic seizures, 5 – turn over into back position, generalized tonic – clonic convulsions according to Racine [25], modified by Becker et al. [2]. Control rats received injection of saline and were kept isolated in the same cages as PTZ-kindled rats for 30 min. Animals considered kindled exhibited stage 4 or (and) 5 of seizures on two consecutive trials. Three groups of animals were selected for further study: the PTZ-treated animals which developed seizures – kindled rats (kindled; $n = 22$); the PTZ-treated animals which did not develop seizures (PTZ, $n = 10$); and the animals repeatedly injected with saline – control rats (control, $n = 15$). The animals usually reached the criterion of kindled seizures after 7–10 injections of PTZ. After reaching the criterion of kindled seizures, 7 days later, the rats were subjected to further behavioral and biochemical testing. After behavioral part of the experiment, the rats received one additional injection of PTZ 3 days after retention test, and 7 days before biochemical analysis.

Water maze test

Training in the maze took place during the light phase of the cycle between 8:00 and 14:00 h. Rats were allowed free access to food and water. A circular pool was used as described by Morris with some modifications [37]. The pool was made of no-brilliant white plastic, 1.20 m in diameter, 0.50 m high and was filled

with 23°C water (0.30 m). Rats were trained to locate a transparent clear Plexiglas platform (10 × 10, 29 cm high) maintained at a fixed location. To render it invisible to the rat, platform was submerged 1 cm below the surface of the water. Animal's backs were marked with black non-toxic paint to facilitate tracking. All rats were subjected to one session of four trials daily for 4 consecutive days. For each trial, the rat was placed in the water facing the wall of the pool at one of three equally spaced starting points, excluding the quadrant with the platform. The order in which these starting points were used was determined randomly for each trial and changed each day to prevent the use of a simple taxis strategy, but the location of the escape platform was always centered in the South East (SE) quadrant. A trial began when the rat was manually placed in the water facing the wall of the pool and was terminated when the rat reached and got on the platform. All rats were left on the platform for 15 s before the next trial was initiated. Rats that failed to find the platform were given a latency score of 60 s. At the end of the day's session the rat was wiped in cloth to dry and was returned to its home cage. In the probe trial on the fifth day of testing, the escape platform was removed from the pool and the rats were allowed to swim for 60 s before the end of the session. Data were recorded by an HVS image analyzing system (Chromotrack, San Diego Instruments) and by videotaping. Data from the water maze included escape latencies to find the platform (s), the velocity (m/s) during trial and the number of crossings of rats over the place where the platform was previously situated (the probe trial).

Biochemical analysis of PTZ concentrations

This experiment was performed on a separate group of naive animals (24 rats, 6 per group). Ten minutes after PTZ administration the animal's brains were rapidly removed and tissue homogenate was prepared by sonification. The brain tissue samples were placed in plastic 1.7 ml Eppendorf tubes containing homogenization solvent (2% HClO₄ with 10 µg/ml 1H-benzotriazole internal standard), sonificated for 30 min and centrifugated at 20,000 rpm for 7 min at 4°C. Then the clear supernatant was collected, filtered through PVDF Durapore 45 µm filter and injected onto the column. PTZ concentration analysis was performed using a modified high-performance liquid

chromatographic (HPLC) method reported previously by Ramzan [27]. The HPLC system consisted of Shimadzu LC-6A pump, Shimadzu CTO-6A oven, Shimadzu SPO-6A spectrophotometric detector (wavelength of 214 nm), Phenomenex Luna C₁₈ 150 mm, 5 µm column equipped with Phenomenex KJO-4282 precolumn. The sample was injected through Rheodyne 7132 injection valve with 20 µl sample loop. The column temperature was 26–27°C. The mobile phase consisted of 5 mM NaH₂PO₄ solution with 12% acetonitrile. During the whole procedure the mobile phase was degassed with helium. The flow rate was 0.3 ml/min. Chromatogram registration and analysis, and construction of calibration curve for PTZ was performed using ChromaX 2000 software. The concentration of PTZ was calculated as µg per gram of brain tissue.

Biochemical analysis of amino acid concentrations

In this experiment, rats were decapitated 10 days after behavioral experiments and their brains were removed and the hippocampus, prefrontal cortex and striatum were dissected bilaterally and frozen at –70°C.

Glutamate, GABA, alanine, serine, histamine and glycine were assayed using a fully automated high-pressure liquid chromatography system with electrochemical detection and standard biochemical methods as reported previously [21]. HPLC analysis was performed using a Luna C₁₈, 25 cm, 5 µm reverse-phase column. Compounds were eluted isocratically with mobile phase delivered at 0.75 ml/min using a Shimadzu Class VP LC 10AD pump. An Antec electrochemical detector ("Intro") with a flow-through cell linked to a Shimadzu Class VP Integrator SCL-10 Avp was used. A high-density glass carbon working electrode (Antec) was operated at +0.85 V. A Rheodyne injection valve with a 20 µl sample loop was used to manually inject the samples. Preparation of the mobile phase and the derivatizing agents were based on the methods described by Rowley et al. [29]. The mobile phase consisted of 0.1 M monosodium phosphate and 0.5 mM EDTA with 25% methanol (v/v) in water adjusted to pH 4.5 with 1 M phosphoric acid. Then, it was filtered through 0.45 µm filters and degassed for 15 min. Stock solutions (0.01 M) of amino acid standards were prepared in double deionized water and kept at 4°C for five days. To prevent adhesion to the glass, GABA standards were prepared

in polyethylene vials. Working solutions were prepared daily by dilutions of the stock solution. To obtain agents for derivatization, OPA (22 mg, Fluka) was dissolved in 0.5 ml of absolute ethanol and 0.9 ml of sodium tetraborate buffer (0.1 M) adjusted to pH 10.4 with 5 M sodium hydroxide. The reaction of derivatization was performed at room temperature. Derivatizing agent (20 μ l) reacted with 1 ml of amino acid standard for 5 min in a polyethylene vial before injection onto the column.

Data analysis

The data are shown as the means \pm SEM. Analysis of variance (ANOVA) for repeated measures was used to assess differences in the latencies (time needed to get to the platform) on four consecutive trial days (Morris maze). LSD *post-hoc* test was performed to identify the origin of any significant differences.

The data from the testing day 5 (Morris maze – crossings on the test day), and the biochemical analysis were analyzed with one-way ANOVA followed by a *post-hoc* test (LSD).

A significance level of 0.05 was the basis of statistical decision. Statistical package Statistica for Windows, release 6 (StatSoft Inc., USA), was used for statistical calculations.

Results

Pharmacokinetic analysis revealed a linear dependence of PTZ concentrations in the brain on PTZ doses indicating a very good blood-brain barrier drug penetration (Fig 1).

Analysis of variance for repeated measures indicated a significant day effect in the acquisitions phase of learning (a latency to find the platform) [$F(3, 78) = 27.05$; $p < 0.001$] (Fig. 2). *Post-hoc* analysis revealed that kindled animals did not differ during consecutive sessions in comparison to the first session ($p > 0.05$). On the other hand, PTZ and control groups showed a significant decrease in latencies in comparison with the first session [PTZ group, second vs. first session, $p < 0.01$, third vs. first session, $p < 0.001$, fourth vs. first session, $p < 0.001$; control group, second vs. first session, $p < 0.05$, third vs. first session, $p < 0.001$, fourth vs. first session, $p < 0.001$]. Effects of neither

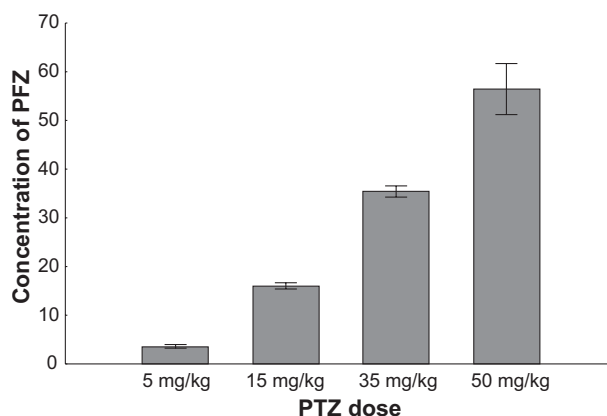


Fig. 1. The concentration of PTZ (g/g of tissue) in the rat brain 10 min after its peripheral administration. The data are shown as the means \pm SEM. The number of rats used in experiment was 24 (6 per group)

group [$F(2, 26) = 1.97$; $p > 0.05$] nor interaction between groups and day [$F(6, 78) = 2.06$; $p > 0.05$], was observed (Fig. 2).

There was a significant effect of a day on the parameter of mean velocity during the acquisition phase of place learning [$F(3, 78) = 6.67$, $p < 0.001$], and the effect of groups per day interaction [$F(6, 78) = 2.74$, $p < 0.05$]. *Post-hoc* analysis indicated that the mean velocity of kindled rats was higher on the first and second day of training ($p < 0.001$ and $p < 0.01$ vs. control, respectively) (Fig. 2).

During the test day (the 5th day), there appeared a significant group effect [$F(2, 26) = 5.60$; $p < 0.05$], and kindled animals spent less time in the quadrant where the platform was previously positioned in comparison with the control ($p < 0.01$) and PTZ group ($p < 0.05$). The velocity of animals did not differ [$F(2, 26) = 1.27$; $p > 0.05$] (Fig. 2).

There were significant differences between groups in the concentration of glutamate in the hippocampus [$F(2, 26) = 3.49$; $p < 0.05$], striatum [$F(2, 26) = 3.50$, $p < 0.05$] and prefrontal cortex [$F(2, 26) = 4.75$, $p < 0.05$] (Fig. 3). *Post-hoc* tests indicated a decrease in glutamate concentration in all examined structures in kindled rats ($p < 0.05$ in the hippocampus and striatum, and $p < 0.01$ in the prefrontal cortex), as well as in the prefrontal cortex in PTZ animals ($p < 0.05$).

There were also statistically significant differences in serine levels in the striatum [$F(2, 26) = 5.54$; $p < 0.01$], and prefrontal cortex [$F(2, 26) = 4.69$; $p < 0.05$]. LSD *post-hoc* test revealed that kindled animals had a decreased striatal concentration of serine ($p < 0.01$),

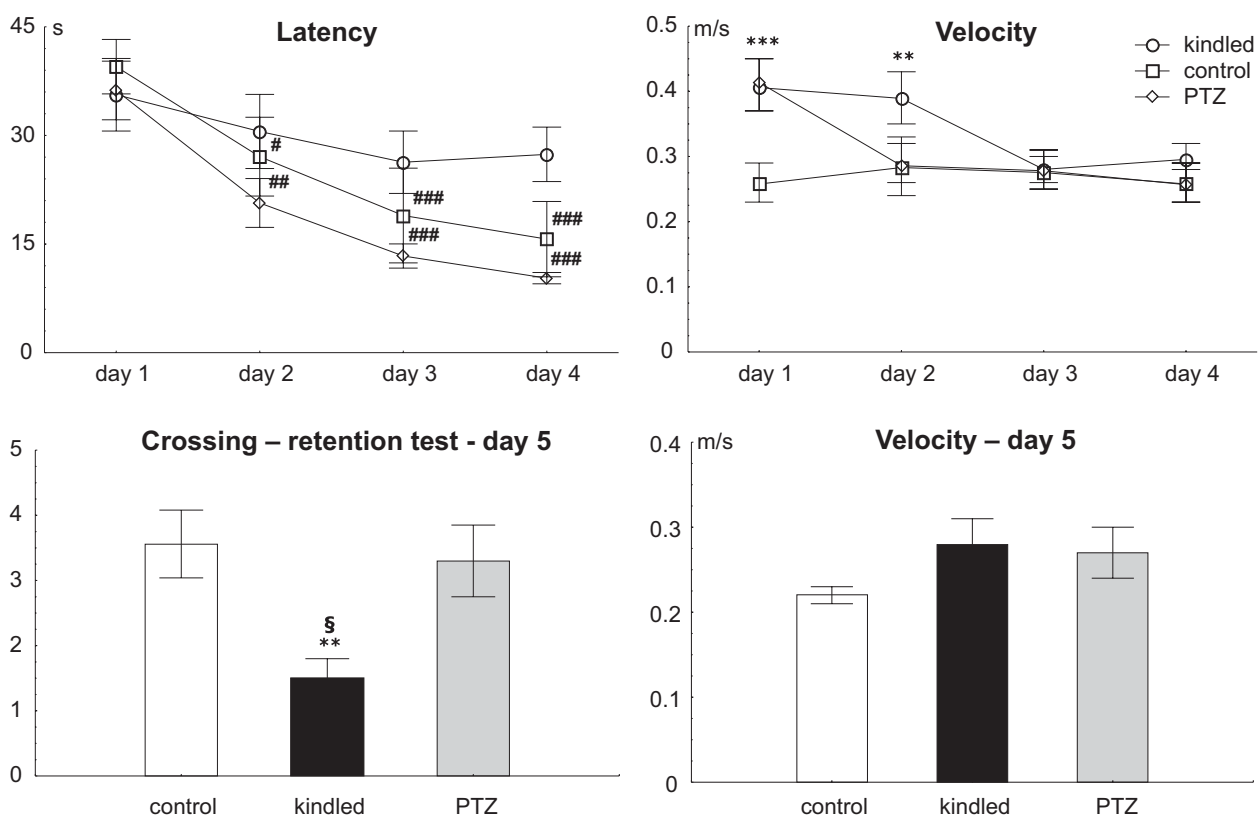


Fig. 2. The effect of PTZ kindling on rat behavior in the Morris water maze test. The data are shown as the means \pm SEM. The number of rats in each group varied from 9 to 10 animals. * $p < 0.01$, ** $p < 0.001$ vs. control group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. DAY 1; § $p < 0.05$ vs. PTZ group. For more explanations see Materials and Methods

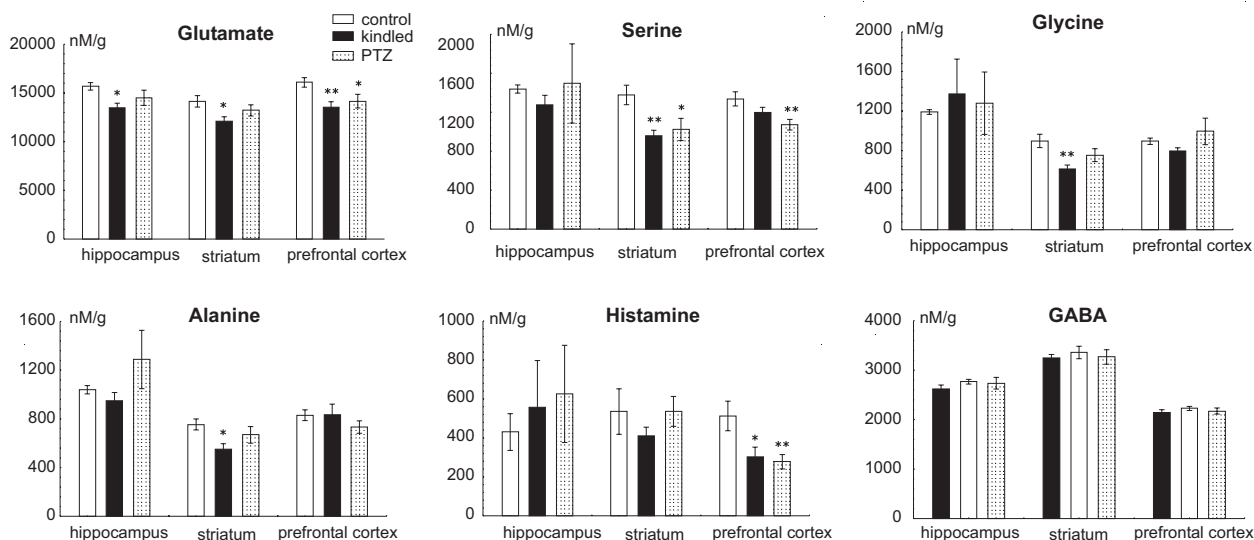


Fig. 3. The effect of kindling on the concentration of amino acids in the brain structures. The data are shown as the means \pm SEM (nM/g of tissue). Open bars – control rats; solid bars – kindled rats; striped bars – PTZ rats. The number of rats in each group varied from 9 to 10 animals. * $p < 0.05$; # $p < 0.01$ vs. control. For more explanations see Materials and Methods

whereas PTZ animals showed decreased levels of serine in the striatum and prefrontal cortex ($p < 0.05$ and $p < 0.01$).

Decreased levels of glycine and alanine in the striatum of kindled animals [$F(2, 26) = 5.81$; $p < 0.01$ and $F(2, 26) = 3.45$; $p < 0.05$, respectively] were also shown (*post-hoc*, $p < 0.01$ and $p < 0.05$ vs. control). Significant differences in histamine concentration were observed in the prefrontal cortex [$F(2,26) = 5.31$; $p < 0.05$]. *Post-hoc* test indicated that kindled and PTZ animals had lower concentration of histamine in comparison with control group ($p < 0.05$ and $p < 0.01$, respectively).

No changes in the brain concentration of GABA across all examined brain structures and experimental groups were observed.

Discussion

The PTZ dose-concentration curve was linear, indicating that the drug penetrated very easily to the brain tissue. This finding confirms the other authors' data [38], and indicates that individual differences in reactivity to the repeated administration of this convulsant agent observed in the present study are not due to the pharmacokinetic processes, but most probably reflect variability in sensitivity to PTZ of particular brain structures or neural circuits.

The most interesting finding of the present study is demonstration of a decrease in learning and memory in the group of kindled animals, accompanied by a selective and long-lasting decline in the brain concentrations of glutamate (in the hippocampus and striatum) as well as of glycine and alanine in the striatum. Accordingly, the latency to reach the hidden platform did not change across training days in kindled rats, indicating a deficit in learning processes. Excitatory amino acids are very well known to modulate memory processes. Particularly important role in this respect is ascribed to the glutamate, NMDA receptor and its co-agonists like glycine and alanine.

Glycine and alanine are known to contribute to the glutamate central effects. Glycine is a well-recognized major inhibitory neurotransmitter in the spinal cord and it also plays an important role as a co-agonist of NMDA subtype of glutamate receptors [13]. Alanine is an important precursor of glutamate, especially dur-

ing recovery from ischemia and hypoxia [1, 7]. Furthermore, it has been recently reported that alanine can also act as a carrier of ammonia nitrogen between glutamatergic neurons and neighboring astrocytes, contributing to neurotoxicity [35]. It was also suggested that similarly to glycine, D-serine and another receptor co-agonist, D-alanine potentiate glutamate neurotransmission *via* selective stimulation of strychnine-insensitive glycine sites at the NMDA receptor [36]. Altogether, it is conceivable, that the present results indicate a dysfunction of glutamatergic system in the brain. The concentration of serine, histamine and GABA were not affected exclusively or significantly in kindled rats, underlining the selectivity of changes in the glutamate, glycine and alanine in the hippocampus and striatum in this group of animals. These findings may be considered as indicating contribution of the NMDA receptor-linked amino acids in the hippocampus and striatum to the cognitive and motor aspects of kindled seizures.

It is possible that a decrease in hippocampal glutamate concentration may account for the decrease in learning and memory in kindled rats, given the important role of this neurotransmitter in the cognitive processes (e.g. long-term potentiation phenomenon), and the key contribution of the hippocampus to the spatial memory [10, 16, 18]. Accordingly, it was previously found that exogenously applied glycine and alanine facilitated the generation of LTP in the CA1 and dentate region of rat hippocampal slices by activating the glycine modulatory sites associated with NMDA receptors [36]. A lower concentration of glutamate and NMDA receptor co-agonists in the brain (glycine and alanine) might indicate, therefore, the general malfunction of central glutamatergic system.

There are several possible explanations for deterioration of cognitive processes, and a decreased concentration of glutamate and related amino acids in the brain of kindled rats. The most parsimonious explanation refers to the degenerative processes in the brain structures, secondary to seizure-related ischemia and hypoxia [22]. Indeed, it has been repeatedly shown that kindled seizures are associated with a selective degeneration of cortical and limbic structures, including hippocampal areas, and involving loss of neurons, neuronal and glial growth, hypertrophy of astrocytes and sprouting of new connections [6, 22, 33]. Accordingly, hippocampus, amygdala and piriform cortex have been reported to have the lowest threshold of stimulation by kindled seizures [14]. Secondly, a de-

crease in glutamate may be due to up-regulation of glutamate transporters [4, 23]. It has been recently demonstrated that the expression of glial and neuronal transporters for glutamate was increased following seizures [8]. This is a general control mechanism responsible for maintaining the physiological concentration of excitatory amino acids and monoamines in the brain. It is possible that kindled seizures stimulate such central feedback processes. However, the results of *in vitro* biochemical analysis indicating a decrease in the concentration of amino acids in brain tissue in a silent period, between fits of seizures suggest that the former explanation is more reliable.

In summary, neuronal damage to the limbic structures and resulting decrease in concentration of excitatory amino acids, may explain the cognitive deficits observed in the examined model of temporal lobe epilepsy.

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