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Benzodiazepine receptor inverse agonist-induced kindling of rats alters learning and glutamate binding

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Abstract

Kindling, recognized as a model of epilepsy, can be obtained by applications of repeated nonconvulsive stimulations that finally lead to generalized seizures. Epileptics often show cognitive impairments. The present work analyzed the learning performance of male Wistar rats kindled with a convulsant inverse agonist of the GABA A-benzodiazepine receptor complex, methyl β-carboline-3-carboxylate (β-CCM). This compound is also known to have an action on learning processes. It was thus interesting to verify if β-CCM kindling had the same impairing action on learning as other kindling agents, such as pentylenetetrazol (PTZ). A two-way active-avoidance shuttle-box learning task was chosen, because a deficit was found after PTZ kindling in this learning model. On the other hand, hippocampal glutamate binding, has previously been shown to be modified by both seizures and learning. Thus, the level of glutamate binding was also measured in the present study. Results showed that fully kindled rats had poorer learning performance after the third day of test than controls or not fully kindled animals. L-[^{3}H] glutamate binding to hippocampal membrane fractions of the fully kindled animals was significantly higher when compared with controls, whereas L-[^{3}H] glutamate binding of not fully kindled subjects did not differ from that of controls. Neuronal plasticity changes are a possible explanation for the correlation between kindling, learning deficits, and increased glutamate binding.

Keywords: β-Carboline; Kindling; Seizures; Learning; Glutamate binding

1. Introduction

Kindling, first described by Goddard et al. [11] as the repeated application of initially subconvulsive electrical stimulation of different brain structures resulting in a progressive development of tonic–clonic seizures, is a recognized model of epilepsy. Kindling can also be obtained by administration of subconvulsive doses of chemicals such as pentylenetetrazol (PTZ) [2,9,19,23,31] and FG 7142, a benzodiazepine receptor inverse agonist [17].

Clinical research has shown that epilepsy can induce emotional and cognitive impairments in patients [8,12,15], especially those with "major motor" and mixed seizure disorders. Cognitive deficits were widely described with the kindling model and using different learning tests [4,29]. PTZ kindling in rats, for example, induces learning impairments and modifications in neuronal plasticity [2].

As far as the transmitter systems involved in this neuronal plasticity are concerned, much attention has been focused on glutamate and on GABA [13], presently known to be the most important excitatory and inhibitory neurotransmitters, respectively, playing a role in the mechanisms underlying kindling. Glutamate is also known to be involved in learning and memory, the LTP phenomenon, and epilepsy [24–26]. Investigations concerning the biochemistry of glutamate, especially modifications in glutamate binding after electrical kindling, showed increased glutamate release and increased receptor density in target neuron populations [28,32,33]. In a comparison of the mechanisms underlying electrical and chemical kindling in PTZ-kindled rats, Schröder et al. [24–26] found an increase in specific L-

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[^{3}H] glutamate binding in hippocampus and in motor, frontal, and inferotemporal cortex. On the other hand, kindling with FG 7142 was not found to induce any significant increase in mRNAs levels for four glutamate receptor subunits in the cortex [16].

To assess the generality of the findings with PTZ, comparison was drawn in the present work with a ligand of a different receptor binding site of the GABA receptor complex, the benzodiazepine receptor inverse agonist methyl β-carboline-3-carboxylate (β-CCM). Effects of different doses of β-CCM have been previously analyzed in mice [6]. Thus, low doses (0.2–0.3 mg/kg) injected acutely improve learning, moderate doses (1 mg/kg) induce anxiety, and higher doses (10 mg/kg) induce seizures. In this study we were interested in establishing a possible relationship between appearance of seizures and their consequences on learning and memory, as well as with modifications in the characteristics of glutamate receptors. For this purpose we first studied the consequences of kindling on learning in Wistar rats. Because β-CCM can improve learning at low doses, it was indeed interesting to verify whether kindling could, in contrast, induce learning deficits similar to those already observed after PTZ kindling [2]. To measure the possible effects on long-term memory, the test previously used in PTZ studies was employed, that is, the two-way active-avoidance shuttle-box. In addition, to evaluate possible variations in L-glutamate binding due to kindling, the density of glutamate receptors in β-CCM-kindled rats was compared with that of control and of acutely treated animals. For these studies, crude membrane fractions of the hippocampus, a brain structure known to play an important role in learning as well as in epilepsy, were used.

2. Materials and methods

2.1. Animals

Experiments were performed on male Wistar rats (aged 8 weeks at the beginning of the experiments). Animals were reared under controlled standard conditions of 20 ± 2°C, a 12-L/12-D photoperiod, with lights on at 0600 h, and relative air humidity of 55–60%. Tap water and commercial rat pellets (Altromin, 326) were available ad lib. Rats were housed in groups of six with dust-free sawdust bedding. For all procedures followed, ethical approval was sought prior to the experiments, according to the requirements of the National Act on the Use of Experimental Animals (Germany).

2.2. Drugs

β-CCM, synthesized by one of us (R.H. Dodd), was dissolved in 0.1 N HCl (75 μl/mg) and diluted to volume with saline. The final pH varied between 2 and 2.4. Injections were given IP in a volume of 1 ml/100 g (body weight). The vehicle was prepared with saline and 0.1 N HCl at the same concentration without β-CCM, thus reaching the same (acidic) pH as the β-CCM solution. This solution is classically used in β-CCM experiments [6] without major behavioral consequences, or visible sign of pain in the animals.

2.3. Chemical kindling

To induce kindling, a 2-mg/kg dose of β-CCM was injected to a group of 14 animals, and a 4-mg/kg dose was injected to a group of 32 animals, IP three times a week for a total of 13 injections in the two different groups.

After each administration behavior was observed for 10 min (β-CCM is known to produce seizures in mice between 50 s and 6 min after injection [6], and the same observation was made in rats in preliminary work). The seizure intensities were classified as follows: stage 0: no response; stage 1: ear and facial twitching; stage 2: convulsive waves through the body; stage 3: myoclonic jerks, rearing; stage 4: turn over onto side position; stage 5: turn over onto back position, generalized tonic–clonic seizures.

2.4. Experimental design

During the kindling study, the β-CCM solution was administered to experimental groups, whereas control groups received the vehicle. Animals were considered to be fully kindled after having reached a mean stage higher than 3.5 over the three last injections. If they did not reach this criterion, animals were not considered to be fully kindled. Learning performance of the rats was evaluated in a two-way shuttle-box starting 24 h after the last (13th) β-CCM or vehicle injection. In the study using 2 mg/kg, 14 rats were tested, for the 4-mg/kg study, 30 rats were used, and the control group consisted of 25 rats. For the binding study four groups were used: four control animals, five not fully kindled, and six fully kindled rats with a 4-mg/kg dose and four animals treated with a single 4-mg/kg dose of β-CCM. As above, animals were considered fully kindled after having reached a mean stage higher than 3.5 over the three last injections stages. If they did not reach this criterion, animals were considered to be not fully kindled.

2.5. Two-way active-avoidance shuttle-box

The automatic shuttle-box (0.25 × 0.25 × 0.6 m) was divided into two identical compartments separated by a 5-cm hurdle. The conditioning stimuli were 40-W bulbs located on the central ceiling of each compartment, and a sound produced by a buzzer. The unconditioned stimulus was an electrical pulse of 0.4–0.8 mA, depending on the individual sensitivity of the animal, delivered through stainless steel rods forming the floor. The conditioned stimuli–unconditioned stimulus were separated by an interval of 4 s. The stimuli were switched off when the rat had changed to

the illuminated goal compartment. One trial was limited to 20 s if the animal failed to react within this period. The mean interval was 30 s. Each session consisted of 30 trials, and was repeated on 4 consecutive days. Sessions were realized between 0800 and 1200 h during the light portion of the 12-L:12-D cycle. Prior to the first session, rats were allowed to explore the box for 5 min, and on the following days for only 1 min. The numbers of escapes (4 s<reaction time<20 s) and conditioned reactions (reaction time<4 s) in each training session were recorded for further evaluation.

2.6. Biochemistry

One week after the last injection animals received a new injection of β-CCM or vehicle and were decapitated 24 h later, and their brains were rapidly removed. Hippocampi were isolated from the rest of the brain using the method described by Popov et al. [22]. Crude membrane fractions were prepared by the method of Zuckin et al. [34], with slight modifications. Briefly, to prepare a 10% homogenate (wet weight/volume), tissue was homogenized in 30 mM Tris–HCl buffer (pH 7.4) containing 2.5 mM $CaCl_2$, and stored for up to 21 days. After thawing, homogenates were centrifuged for 20 min at 50,000 × *g*. The resulting pellets were washed three times with homogenization buffer and centrifuged again. The L-[^{3}H] glutamate (specific activity: 1.43 TBq/mmol, NEN, Germany) binding was measured using a modified method initially described by Baudry and Lynch [1]. Specific binding was calculated by subtracting nonspecific binding, defined as that observed in the presence of 50 nM L-[^{3}H] glutamate plus 100-μM unlabeled L-glutamate (Serva), from total binding, obtained with 50 nM L-[^{3}H] glutamate alone.

The pellet was suspended in Tris–HCl buffer. Aliquots (50 μl) of the crude membrane suspension containing 150–250 μg of protein were mixed with 50 nM L-[^{3}H] glutamate specific activity, to a final volume of 1 ml, and incubated for 40 min at 37°C. All assays were performed in duplicate. The reaction was terminated by rapid filtration under reduced pressure through GF/A glass fiber filters. Filters were washed three times with 3 ml of buffer each time, and taken up in 10 ml of a toluene-containing solvent for liquid scintillation counting. The protein content was determined according to the method described by Lowry et al. [18].

2.7. Statistics

Comparisons of regression slopes of 4-mg/kg kindling development curves were estimated with a covariance analysis, with the day as a covariance factor. For the shuttle-box, the repeated-measures analysis of variance was used, with between-factor groups and within-factor days. This analysis was followed by partial comparisons of adjusted means. Significance level was first determined as $p = 0.05$, but because nine group–day comparisons were made for each type of tested group, the significance level was divided by nine to set the significance level at $p = 0.005$. To determine the significance of the binding measurements, a non-parametric test was used. For general comparison the Kruskal–Wallis test was used, and to compare two groups the Wilcoxon two-sample test was used.

3. Results

3.1. β-CCM kindling

A first experiment in rats was performed using a dose of 2 mg/kg already tested in mice (results not pub-

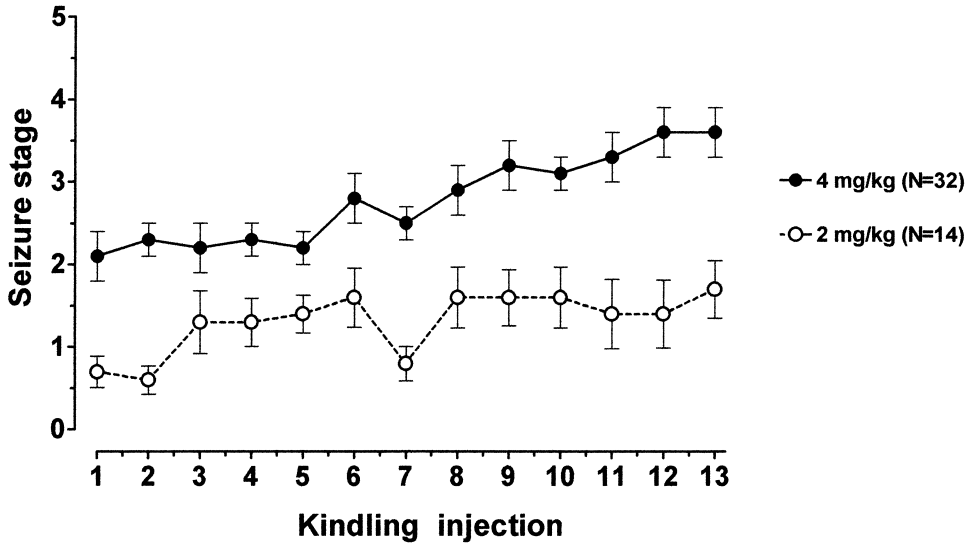

Fig. 1. Kindling development induced by different doses (2 and 4 mg/kg) of β-CCM. Seizure stages (mean ± SEM) as a function of number of injections (13) (for statistics, see text).

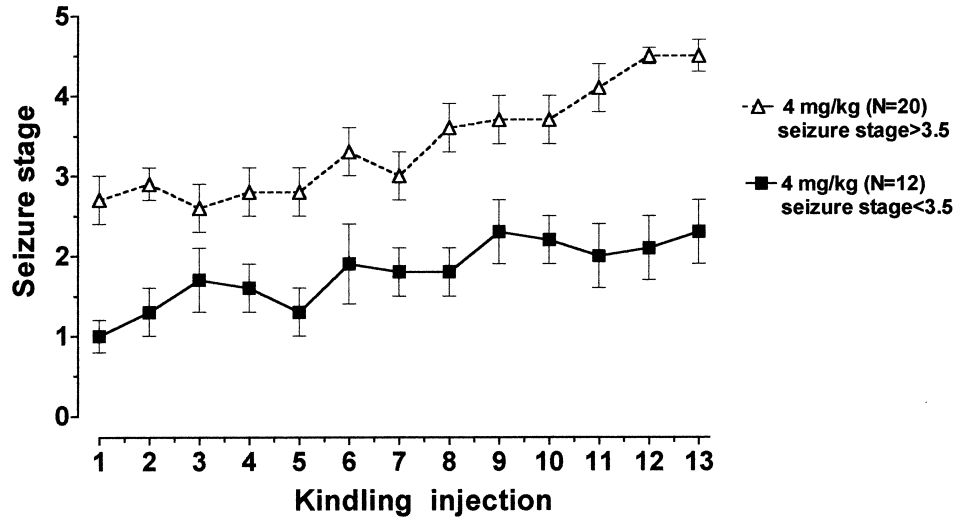

Fig. 2. Kindling development induced by a 4 mg/kg dose of β-CCM. Animals divided according to the kindling stage. Seizure stages (mean ± SEM) as a function of number of injections (13) (for statistics, see text).

lished). Rats treated as such never reached seizure stages higher than 1.7; the kindling criterion (stages higher than 3.5 on the three last injections) was thus not reached (Fig. 1). The regression slopes of this group and of the group of animals treated with 4 mg/kg were not different (Fig. 1). With a dose of 4 mg/kg, some animals reached kindling criterion after thirteen injections. For this reason these 4 mg/kg injected animals were divided into two groups, depending on the kindling success, one with a kindling stage lower than 3.5 (n=12) considered as "not fully kindled" and one higher than 3.5 (n=20) where all rats were considered to be fully kindled (Fig. 2). Kindling regression slopes for the nonkindled group (seizure stage <3.5) was 0.089 (H0: slope=0 — p=0.0004) and for the fully kindled animals (seizure stage >3.5) 0.163 (H0 — p=0.0001). Although the start point of seizure activity seems different, the regression slopes of these two groups were significantly different, $F(1,382)=5.41$, $p=0.0206$, demonstrating the more pronounced kindling development in the fully kindled group. In the not fully kindled group, because the level of seizures remains very low after 13 injections, no attempt was made to continue with further injections.

3.2. Shuttle-box

Rats injected with 2 mg/kg β-CCM never reached our kindling criterion (Fig. 1). Similarly, there was no difference between control animals and those injected with the 2-mg/kg dose in learning performance as evaluated in the shuttle-box test, $F(1,37)=1.53$, $p=0.2234$ (Fig. 3).

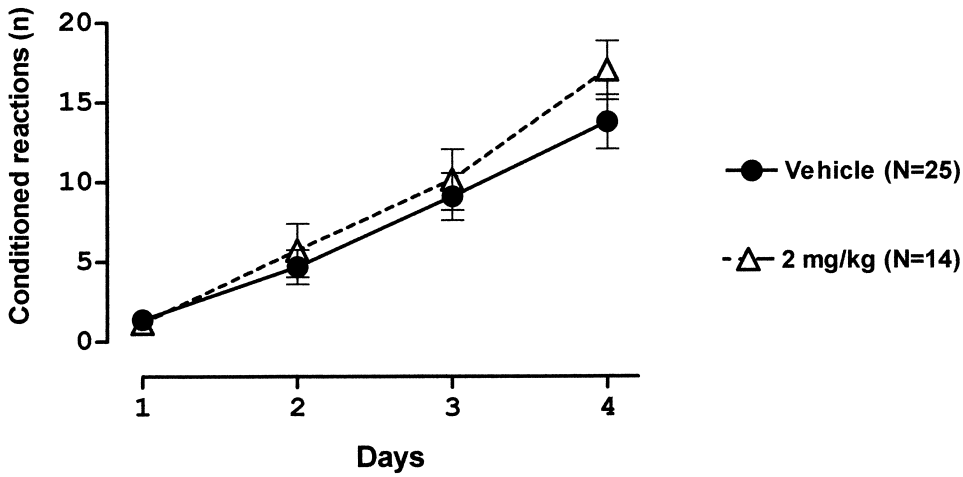

Fig. 3. Learning performance of kindled rats in the shuttle-box test using 2 mg/kg β-CCM. Conditioned reactions (mean ± SEM) are given for four training sessions.

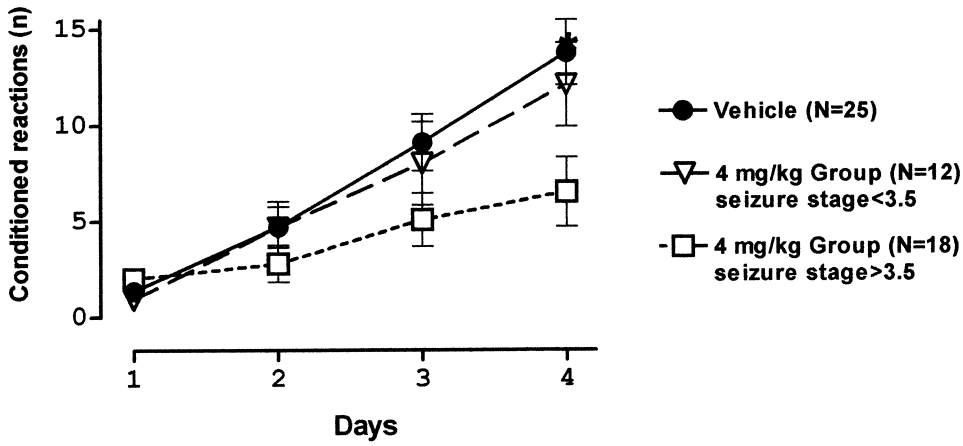

Fig. 4. Learning performance of kindled rats in the shuttle-box using 4 mg/kg β-CCM. Rats were kindled by 4 mg/kg β-CCM and the group of kindled rats was divided according to whether they reached kindling state. Conditioned reactions (mean ± SEM) are given for four training sessions. * $p<0.005$.

When comparing the learning performance to acquire the conditioned reaction in the shuttle-box, the two groups injected with the 4-mg/kg dose (the group with seizure stage <3.5 and the one with the seizure stage >3.5) as well as the control group showed a significant general effect of group, $F(2,48)=5.99$, $p=0.0048$, and day, $F(3,160)=23.59$, $p=0.0001$ (Fig. 4). Partial comparison showed no differences between the three groups the first 2 days. A slight but not significant impairment of performance of fully kindled animals, compared to control animals, was observed the third day ($p=0.0296$, NS) at the defined criterion of $p=0.005$. The impairment of the fully kindled group became clearly significant by the last day, compared to the performance of the control group ($p=0.0001$).

Learning performance of rats with seizure stage <3.5 did not differ significantly from that of control animals.

3.3. Binding kinetics of L-[^{3}H] glutamate to hippocampal crude synaptosomal membranes

As mentioned earlier, comparison was made among control animals, fully kindled (seizure criterion >3.5), and not fully kindled (seizure criterion <3.5) rats (4 mg/kg β-CCM) and acutely treated animals (4 mg/kg β-CCM).

There was a slight but not significant increase in specific glutamate binding for the not fully kindled group (4 mg/kg) and acutely treated animals compared to the control group (Fig. 5) (not fully kindled: $z=-1.347$, NS; acutely treated: $z=-0.722$, NS).

In contrast, the specific L-[^{3}H] glutamate binding to hippocampal membrane fractions of the fully kindled animals was significantly increased in hippocampus when compared with controls ($z=-2.0254$; $p=0.0428$).

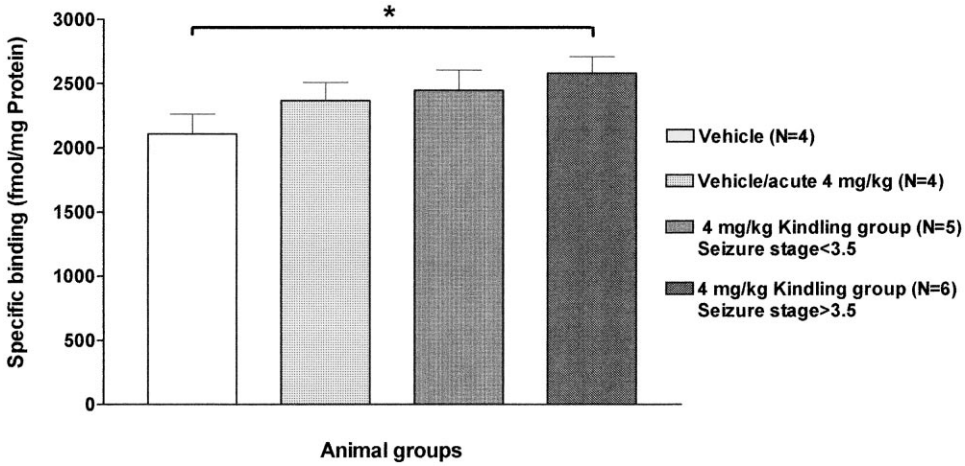

Fig. 5. Specific glutamate binding study.Specific glutamate binding (mean ± SEM, fmol/mg protein) for the four different groups: controls (vehicle treated), controls+one acute β-CCM injection (vehicle/acute), treated group with seizure stage< 3.5, treated group with seizure stage >3.5; *$p<0.05$.

4. Discussion

Several studies have been devoted to behavioral, neurophysiological, and neurochemical changes after kindling. These changes are long-lasting trans-synaptic modifications generated in different brain regions, with modification in neurotransmission leading to an increased excitability in animals [20,32]. The mechanisms underlying kindling are nowadays still not completely understood. On the other hand, several impairments of cognitive processes in animals have been described after kindling [29,31]. In epileptic humans, intellectual, emotional, and psychosocial impairments have been described [8].

The present results demonstrate that kindling induced by repeated administration of a benzodiazepine receptor inverse agonist also leads to cognitive impairments. These results are similar to those found with PTZ promoted kindling. It can be assumed that both PTZ and β-CCM, which modulate the GABA receptor by interacting with different binding sites, can induce similar kindling states. It should also be mentioned that these consequences of kindling on cognition do not follow the dose graduation described for single injections by Chapouthier and Martin [6]. Indeed, in the fully kindled state, there is no longer this relationship between a specific dose and the corresponding behavior [6].

In animals, former investigations have shown that a single injection of PTZ, inducing one acute generalized convulsion, had no consequence on short-term and long-term memory tested 24 h later [2]. PTZ kindling also did not modify short-term memory [2]. In the shuttle-box experiment of the present work, controls and not fully kindled animals show normal and identical learning abilities. No difference between these two groups and the fully kindled animals was observed during the first 2 days of training. Animals fully kindled with 4 mg/kg β-CCM showed, however, a significant deficit in shuttle-box performance after the third day of testing, suggesting disruption of memory storage and an impaired long-term memory.

Furthermore, other data reported in the literature described similar learning (acquisition) deficits, in a water-maze task, in rats kindled in the hippocampal field CA1 and reaching generalized seizures, but not in rats partially kindled or not reaching generalized seizures [10]. Amygdala kindling and kindling of the dorsal hippocampus significantly induced deficits in the retention of brightness discrimination in a Y-maze in kindled rats compared to control animals [2]. Electrical kindling in the olfactory bulb also induced deficits in long-term memory in the radial arm maze test [30]. These last results seem to confirm our observations in the shuttle-box test after PTZ [2] or β-CCM (the present study) kindling: during the first days of testing there is no difference between fully kindled and normal animals in their aptitude to perform the task, whereas a few days later, a difference appears.

Previous studies have provided evidence for a correlation between increase of frequency of seizure events induced by kindling and progressive neuronal loss in some brain structures [5]. In human epilepsy [14] as well as in animal kindling-induced seizures [3], neuronal loss is essentially localized in hippocampal regions such as the gyrus dentate, CA1, CA3, and sometimes, but very rarely, in CA2 and in some limbic structures [5]. One hypothesis that can explain memory deficits is thus based on neuronal loss. Many results seem to confirm a correlation between hippocampal lesions and memory impairments [21]. Neuronal loss has also been observed after PTZ kindling in CA1 region [3] and after electrical kindling [27]. In the present study, however, cell loss was not measured, but this could, nevertheless, be a cause of the observed memory impairments.

Increase in glutamate binding in hippocampus after PTZ kindling [24–26] has also been observed after β-CCM kindling in the present study, and probably reflects changes in receptor density. In not fully kindled animals (seizure rate <3.5) and in rats subjected to a single injection, no significant changes in glutamate binding were induced. These results seem to confirm possible correlations between repetition of administration of subconvulsive doses, increased susceptibility to injection leading to the development of repeated tonic–clonic seizures, and enhancement in glutamate receptor density. A fully kindled state is thus necessary for specific alterations of the density of glutamate binding sites. The injections leading to full kindling could induce plastic changes in synaptic connectivity: neuropharmacological analysis of PTZ-induced kindling in mice also showed a significant increase in the specific binding of [^{3}H] glutamate in the cerebral cortex membranes of fully kindled mice [7]. However, other studies of FG 7142 kindling did not provide evidence for significant increase in mRNA levels of four glutamate receptor subunits in the cortex [16]. Further experiments should explore which specific receptor subtypes and subunits might be modified by the kindling protocol used in this work.

In conclusion, our data show a strong correlation between a behavioral expression of β-CCM kindling (repeated seizures), learning deficits and an increase in glutamate binding in Wistar rats. Neuronal plasticity changes resulting from these repeated administrations are a likely explanation for the impairments observed in learning.

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