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Epilepsy and hippocampal neurodegeneration induced by glutamate decarboxylase inhibitors in awake rats

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ABSTRACT

Glutamic acid decarboxylase (GAD), the enzyme responsible for GABA synthesis, requires pyridoxal phosphate (PLP) as a cofactor. Thiosemicarbazide (TSC) and γ-glutamyl-hydrazone (PLPGH) inhibit the free PLP-dependent isoform (GAD65) activity after systemic administration, leading to epilepsy in mice and in young, but not in adult rats. However, the competitive GAD inhibitor 3-mercaptopropionic acid (MPA) induces convulsions in both immature and adult rats. In the present study we tested comparatively the epileptogenic and neurotoxic effects of PLPGH, TSC and MPA, administered by microdialysis in the hippocampus of adult awake rats. Cortical EEG and motor behavior were analyzed during the next 2 h, and aspartate, glutamate and GABA were measured by HPLC in the microdialysis-collected fractions. Twenty-four hours after drug administration rats were fixed for histological analysis of the hippocampus. PLPGH or TSC did not affect the motor behavior, EEG or cellular morphology, although the extracellular concentration of GABA was decreased. In contrast, MPA produced intense wet-dog shakes, EEG epileptiform discharges, a >75% reduction of extracellular GABA levels and remarkable neurodegeneration of the CA1 region, with >80% neuronal loss. The systemic administration of the NMDA glutamate receptor antagonist MK-801 30 min before MPA did not prevent the MPA-induced epilepsy but significantly protected against its neurotoxic effect, reducing neuronal loss to <30%. We conclude that in adult awake rats, drugs acting on PLP availability have only a weak effect on GABA neurotransmission, whereas direct GAD inhibition produced by MPA induces hyperexcitation leading to epilepsy and hippocampal neurodegeneration. Because this degeneration was prevented by the blockade of NMDA receptors, we conclude that it is due to glutamate-mediated excitotoxicity consequent to disinhibition of the hippocampal excitatory circuits.
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1. Introduction

GABA is the most important inhibitory neurotransmitter of mammalian CNS. It is synthesized from glutamate by the pyridoxal-5′-phosphate (PLP)-dependent enzyme glutamate decarboxylase (GAD, EC 4.1.1.15). Early studies on the kinetics and regulation by PLP of GAD activity suggested the existence of two forms of the enzyme with different affinity for the coenzyme and cellular localization (Bayón et al., 1977a,b). These two forms were later identified and characterized as GAD65 and GAD67, according to their molecular weight, with different functions related to PLP affinity (Erlander et al., 1991; Esclapez et al., 1994; Kaufman et al., 1991), and studies with the isolated GAD65 and GAD67 isoforms confirmed that free PLP activates GAD65, located mainly in nerve endings, by the conversion of the apoenzyme to the holoenzyme form, whereas the cytosolic GAD67 behaves as an holoenzyme with bound PLP (Battaglioli et al., 2003; Kaufman et al., 1991; Martin et al., 1991).

In some cases of epilepsy in infants, vitamin B_6 deficiency has been related to convulsions because PLP (the phosphorylated form of pyridoxal, active form of vitamin B_6) levels in CSF are decreased and seizures disappear after vitamin B_6 administration (Coursin, 1969; Goyal et al., 2013; Veerapandiyan et al., 2011). Experimental animal models of PLP-dependent epilepsy had been generated in rodents by a pyridoxine-deficient diet, which produced spontaneous epileptogenic discharges ten weeks after starting the diet (Sharma and Dakshinamurti, 1992), or by using drugs to interfere with PLP function, as the carbonyl-trapping hydrazides (for reviews see Tapia, 1975, 1983). Among them, thiosemicarbazide (TSC) produces GAD inhibition and convulsions in rodents (Abe and Matsuda, 1979; Killam and Bain, 1957; Dunlop et al., 1960). In mice, the PLP-dependent GAD activity is also inhibited when PLP in brain is decreased by blockade of pyridoxal kinase activity after the systemic administration of γ-glutamyl-hydrazone (PLPGH), and these changes have been correlated with the occurrence of

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convulsions (Massieu et al., 1994; Pérez de la Mora et al., 1973; Tapia and Awapara, 1967, 1969; Tapia et al., 1969; Tapia and Pasantes, 1971). However, microinjection of TSC or PLPGH into the substantia nigra reticulata or the hippocampus of adult anesthetized rats, although both significantly decreased GAD activity, did not result in behavioral alterations (Tapia and Salazar, 1991).

Other inhibitors known to inhibit GAD activity and interfere with the GABAergic system are mercaptoacids, which compete with glutamate as a substrate for GAD (Wu and Roberts, 1974). Among these, the most powerful is 3-mercaptopropionic acid (MPA), which inhibits GAD in rat (Lamar, 1970; Rodríguez de Lores Arnaiz et al., 1973) and mouse (Horton and Meldrum, 1973) brain homogenates, and induces convulsions after the systemic administration in rat (Karlsson et al., 1974; Lamar, 1970; Netopilova et al., 1997), mouse (Löscher, 1979) and baboon (Horton and Meldrum, 1973).

In the present study, we tested if the GAD inhibitors TSC, PLPGH and MPA can cause epilepsy after their perfusion during several min by reverse microdialysis in the hippocampus of awake rats, in order to avoid the effect of the anesthetics. In addition, the second aim of the present study was to establish whether the neuronal hyperexcitation resulting from a decrease of GABAergic neurotransmission after GAD inhibition could induce hippocampal neurodegeneration, as well as the possible involvement of glutamate-mediated excitotoxicity, since we have previously shown that stimulation of glutamate release leads to such neurodegeneration (Peña and Tapia, 2000). For these purposes we administered the GAD inhibitors and a glutamate receptor blocker and correlated the behavioral alterations with EEG changes, followed by histological analysis of the tissue to assess neurodegeneration. Our results show that drugs acting on PLP availability do not induce hyperexcitation, while MPA produced epilepsy and neurodegeneration through an excitotoxic mechanism.

2. Materials and methods

2.1. Microdialysis and EEG recording

All drugs, except PLPGH which was synthesized from L-glutamic acid-γ-hydrazide and PLP as previously described (Tapia and Awapara, 1969; Tapia et al., 1969), were purchased from Sigma (St. Louis, MO). Adult male Wistar rats (220–240 g) were used and handled in accordance with the international standards of animal welfare and to the Institutional Committee for the Care and Use of Laboratory Animals (Approval No. RTI-121-14).

The surgery for the cortical EEG recording and the microdialysis procedure in awake freely moving rats was carried out as previously described (Vera and Tapia, 2012). Briefly, rats were anesthetized with 0.5–2% isoflurane in 95% O_2/5% CO_2 mixture and mounted in a stereotaxic apparatus. Two electrode screws were fixed with dental cement on the cranial area over the parietal cortex for the EEG recording. A Grass polygraph with a low frequency filter at 3 Hz and a high frequency filter at 100 Hz was used. The latency to the onset of the first epileptiform discharge as well as the frequency and duration of the epileptiform discharges were determined. Duration of discharges was measured from the beginning of the hypersynchronic activity to the last high-amplitude spike in a discharge train. For microdialysis, a cannula guide was implanted and fixed with dental cement in the right hippocampus (coordinates from bregma: AP −3.6, L −2.1, and V −4.0; Paxinos and Watson, 1982). After waking from anesthesia, animals were transferred to individual cages with food and water ad libitum. Five days later, rats were placed in a bowl of a free movement microdialysis system (CMA 120) and perfused through a microdialysis cannula (CMA/12 membrane with 2 mm length and 0.5 mm diameter CMA/Microdialysis, Acton, MA, USA) inserted into the cannula guide, at a flow rate of 2 μL/min with Ringer–Krebs medium containing (in mM): 118 NaCl, 4.7 KCl, 1.18 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$ and 10 glucose (pH 7.4). After 1 h of stabilization, three fractions of 25 μL (12.5 min) were collected for measuring the basal extracellular amino acids. Then, GAD inhibitors were perfused during 62.5 min and five 25 μL fractions were collected for the measurement of amino acids, followed by Krebs medium for 37.5 min.

Based on our previous study in which intranigral or intrahippocampal rapid microinjection of 0.2 μL of 200–800 μM PLPGH and TSC in anesthetized rats (Tapia and Salazar, 1991) did not induce hyperexcitation, we initially perfused these drugs at 1 mM and 5 mM concentrations during 62.5 min with no significant effects. We then increased the concentration to 15 mM TSC and 25 mM of PLPGH, which produced some spikes on the EEG recording. In the case of MPA, based on previous studies showing that its systemic or intracerebroventricular administration produced convulsions, GAD inhibition and decreased GABA levels in brain (Fan et al., 1985; Netopilova et al., 1997), we tested the perfusion of 0.6, 3.0 and 6.6 mM MPA. With the highest dose used 4 out of 8 rats died in tonic convulsions within 30–50 min; the four surviving rats showed continuous clonic–tonic movements and were incapable of walking during the following hours and were therefore euthanized, so with this dose of MPA we show only the effects of wet-dog shakes and EEG recording during the initial 2 h.

In order to test the effect of the NMDA receptor antagonist MK-801, the drug was injected i.p. 30 min before the administration of 3 mM MPA, at a dose of 1 mg/kg, which has been previously shown to be effective against the glutamate-mediated epilepsy and neurodegeneration induced by 4-aminopyridine (Ayala and Tapia, 2005; Peña and Tapia, 2000). A control group of rats received only MK-801.

The behavior of the animals was observed during the following 3–4 h, with continuous EEG recording.

2.2. Amino acids analysis

The concentration of aspartate, glutamate and GABA in the microdialysis fractions was measured by HPLC in a Beckman liquid chromatograph after derivatization with *o*-phthaldialdehyde, as previously described (Peña and Tapia, 2000).

2.3. Histology

Twenty-four hours after the experiment, animals were anesthetized with pentobarbital and transcardially perfused with 0.9% saline, followed by 10% formaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed, postfixed and transferred to 10%, 20% and 30% sucrose gradient during 24 h each. Brain coronal sections (40 μm thick) were obtained in a cryostat and stained with cresyl violet for morphological determinations. Hippocampal healthy neurons from both brain hemispheres were identified as those of >15 μm diameter, with clear cytoplasm, similar to those of control rats. Cells were counted with a 20× microscopy objective (30,000 μm^2), with the help of an image analyzer system (NIH Image 1.6). Three brain slices from each rat, in which the cannula tract was evident were selected for neuronal counting; we show only the results of the CA1 region, because no changes were observed in other hippocampal areas.

2.4. Statistical analysis

Statistical comparison was carried out using analysis of variance (two-way ANOVA-Tukey or two-way ANOVA-Bonferroni). $p < 0.05$ was considered statistically significant, as indicated in the figures.

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