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Non-competitive antagonists of NMDA and AMPA receptors decrease seizure-induced c-fos protein expression in the cerebellum and protect against seizure symptoms in adult rats

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ABSTRACT

The aim of the present study was to examine the role of ionotropic glutamate receptors in the cerebellum during generalized seizures. Epileptic neuronal activation was evaluated through the immunohistochemical detection of c-fos protein in the cerebellar cortex. Generalized seizures were precipitated by the intraperitoneal injection of 4-aminopyridine. The animals were pretreated with the NMDA receptor antagonists MK-801 (2 mg/kg), amantadine (50 mg/kg), and the AMPA receptor antagonist GYKI 52466 hydrochloride (50 mg/kg). Two hours after 4-aminopyridine injection, the number of c-fos immunostained cell nuclei was counted in serial immunohistochemical sections of the cerebellar vermis. The number of c-fos immunostained cell nuclei in the granular layer decreased significantly in animals pretreated with the glutamate receptor antagonists compared to the untreated animals having convulsion. We can conclude that mossy fiber stimulation exerts its seizure-generating action mainly through the ionotropic glutamate receptors of the mossy fiber synapses. Both NMDA and AMPA receptor antagonists are effective in reducing glutamate-mediated postsynaptic effects in the cerebellar cortex.

1. Introduction

Epilepsy causes distinct neuropathological alterations in the cerebellum resulting in the significant loss of Purkinje cells (Honavar and Meldrum, 2002). Nevertheless, there are epileptic seizures which clearly originate from pathological lesions of the cerebellum (Boop et al., 2013). Moreover, stimulation of the cerebellum is used to treat intractable epilepsy (Ge et al., 2013). The double-faced role of the cerebellum in epilepsy can be explained by the double-faced anatomical connections of the cerebellum and the neocortex-diencephalon axis. The neocortex is projecting to the pontine nuclei, which project to the cerebellum through the pontocerebellar tract (Lee and Mihailoff, 1990). Axon terminals of the corticopontine tract make excitatory synaptic contacts with the dendrites of the pontine neurons (Brodal and Bjaalie, 1992). The glutamatergic pontocerebellar system terminates as mossy fibers in the granular layer of the cerebellar cortex stimulating mainly granule cells and Golgi cells (Castejon and Castejon, 2000). The cerebellar efferent information flows to the deep cerebellar dentate nucleus, which projects to the thalamus, and the thalamic neurons project to the frontal neocortical areas in order to form excitatory synapses (Geminiani et al., 2017). The dentate nucleus is also connected to the pontine nuclei (Lee and Mihailoff, 1990).

In our previous studies, we proved that generalized tonic–clonic seizures (GTCS) precipitated by the potassium channel blocker 4-aminopyridine (4-AP) caused long-lasting, significant increase of c-fos protein expression in the granular layer of the cerebellar cortex (Tóth et al., 2015), very similarly to that seen in the hippocampus and neocortex during seizure (Mihály et al., 2001; 2005). We also demonstrated through immunohistochemistry and Western blotting methods that the transection of the middle cerebellar peduncle (MCP) significantly decreased c-fos expression in the animals with seizure (Tóth et al., 2015). These findings suggested the primary importance of the mossy fibers in cerebellar seizure initiation (Tóth et al., 2015). Mossy fibers of the MCP use glutamate as a neurotransmitter (Somogyi et al., 1986). Accordingly, granule cells in the cerebellar cortex have NMDA and AMPA type ionotropic glutamate receptors (Limatola, 2003; Sanchez-Perez et al.,

Abbreviations: AOI, area of interest; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, analysis of variance; 4-AP, 4-aminopyridine; c-fosIR, c-fos immunoreactive; DAB, 3, 3′-diaminobenzidine tetrahydrochloride; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetic acid; GABA, γ-amino butyric acid; GTCS, generalized tonic–clonic seizure; GYKI 52466 or GYKI 52466 hydrochloride, 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride; IgG, immunoglobulin G; i.p., intraperitoneal; MCP, middle cerebellar peduncle; MK-801, dizocilpine; NMDA, N-methyl-D-aspartic acid; NR1,2,3, NMDA receptor subunits; PAP, peroxidase-anti-peroxidase; PBS, phosphate buffered saline

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2005; Koutsouraki et al., 2013). Both NMDA and AMPA receptors are able to induce the expression of transcription factors through strong Ca^{++} influx (Rogawski, 2013; Rothan et al., 2017). This ionotropic glutamate transmission mechanism explains our observations of the significant increase in c-fos protein in the cerebellar cortex in 4-AP seizures (Tóth et al., 2015). The pivotal role of the mossy fibers in this transcription factor expression has been proven through the transection of the MCP, which reduced the ipsilateral c-fos expression by 80% (Tóth et al., 2015). MCP transection has also affected the contralateral cerebellar hemisphere, and the seizure-generated c-fos expression has decreased by 30% in the contralateral hemisphere, as detected by Western blotting and immunohistochemistry (Tóth et al., 2015).

Ionotropic NMDA type glutamate receptor antagonist MK-801 (dizocilpine) is a selective, non-competitive antagonist, which has anticonvulsant and neuroprotective effects as well (Wong et al., 1986; Braitman and Sparenborg, 1989; Szakács et al., 2003). The low-affinity NMDA receptor blocker amantadine (1-aminoadamantane) has anticonvulsant and neuroprotective roles as well (Kornhuber et al., 1994; Szakács et al., 2003). GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride) is a selective, non-competitive antagonist of the AMPA-type glutamate receptors (Donevan and Rogawski, 1993; Arai, 2001; Weiczner et al., 2008). Previous experiments performed in our laboratory proved that seizure-induced c-fos protein expression in the neocortex and hippocampus could be significantly inhibited by MK-801, amantadine, and GYKI 52466 pretreatments (Szakács et al., 2003; Weiczner et al., 2008). Closer investigation of the different receptor blockers revealed different action mechanisms in case of the NMDA (Zádor et al., 2008) and AMPA receptor antagonists (Weiczner et al., 2008). The aim of the present study was to measure the net changes in c-fos expression of cerebellar granule cells in the 4-AP acute seizure model following pretreatment with MK-801, amantadine, and GYKI 52466, and to compare their effectiveness on local neuronal activation (c-fos expression) and on the complex behavioral symptoms of the 4-AP seizures.

2. Material and methods

Adult, male, 220–240 g (12–14-week old) Wistar rats were housed in a light and a temperature-controlled room and had free access to food and water. The animals were kept and the experiments were conducted in accordance with prevailing laws and ethical considerations of the European Union (European Community Council Directive; 2010/63/EU). Written permission for the protocols of the experiments was obtained from the Ethics Committee for the Protection of Animals in Research of the University of Szeged. Control, untreated animals (4 rats) received 1 ml physiological saline intraperitoneally. The seizures were induced with a single intraperitoneal (i.p.) injection of 4-aminopyridine (4-AP; Sigma-Aldrich, St. Louis, MO, USA) dissolved in physiological saline (5 mg/kg 4-AP, 0.67 mg/ml 4-AP concentration). In previous investigations, this dose proved to be epileptogenic (Mihály et al., 1990, 2001,2005; Tóth et al., 2015). The rats treated with 4-AP displayed GTCS (ly et al., 1990, 2001;). The NMDA receptor antagonists MK-801 and amantadine (Sigma, St. Louis, MO) were dissolved in physiological saline. The GYKI 52466 hydrochloride (Sigma, St. Louis, MO) was dissolved in 50% DMSO (DMSO: dimethyl sulfoxide; Sigma, St. Louis, MO; de Sarro et al., 1995; Weiczner et al., 2008) in physiological saline. The 4-AP-treated animals were divided into six groups with four animals in each group (altogether 24 animals). In the first set of three groups, the animals were pretreated with glutamate receptor antagonists MK-801 (2 mg/kg), amantadine (50 mg/kg), and GYKI 52466 hydrochloride (50 mg/kg). After the pretreatment (15 min following the administration of the antagonist), the convulsant 4-AP was administered intraperitoneally (dose: 5 mg/kg). In the second set of three groups, the animals received the solvent of the antagonists, and 15 min later, the 4-AP. The experiments were finished 2 h after the 4-AP injection. The symptoms during seizure were observed, and the latency of the first GTCS was measured. At the end of the observation (2 h following the 4-AP injection), the rats were deeply anesthetized with diethyl-ether (Fluka). The chest was opened, the aorta was cannulated, and the animals were perfused with fixative, which contained 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After the transcardiac perfusion, the brain was dissected and postfixed in the same fixative for 1 h at room temperature. The vermis was separated and soaked in 20% sucrose solution (20% sucrose in 0.1 phosphate buffer, pH 7.4.) for 10–12 h. Sagittal plane frozen sections (24 µm thickness) were cut on a freezing microtome and used as free-floating sections in immunohistochemistry.

2.1. Behavioral studies

Ten adult male Wistar rats were used in every group (40 animals; each 230 g, 13 weeks old). The NMDA receptor antagonists MK-801 and amantadine (Sigma, St. Louis, MO) were dissolved in saline, whereas GYKI 52466 hydrochloride (Sigma, St. Louis, MO) was dissolved in 50% DMSO in saline (de Sarro et al., 1995; Weiczner et al., 2008). The seizures were induced with a single i.p. injection of 4-AP, which was dissolved in physiological saline (5 mg/kg 4-AP in 0.67 mg/mL vehicle), and this 4-AP dose proved to be epileptogenic (ly et al., 1990, 2001; ; Tóth et al., 2015). The animals were pretreated with glutamate receptor antagonists MK-801 (2 mg/kg), amantadine (50 mg/kg), and GYKI 52466 (50 mg/kg) via i.p. injection. After the pretreatment (15 min later), the convulsant agent 4-AP was administered i.p. (5 mg/kg). In the control group, the animals received the solvent of the antagonists (50% DMSO in 0.9% NaCl solution) and the 4-AP, and the seizure symptoms were registered: the latency of the first GTCS was measured from the time of the 4-AP injection. The appearance of the first GTCS and the number of the animals which displayed the GTCS were assessed (Table 1). The latencies of the first GTCS were statistically investigated with one-way analysis of variance (ANOVA), whereas the incidence of the generalized tonic–clonic seizure was analyzed by Fisher's exact test using SPSS 9.0 statistical software. During the analysis, the pretreated groups were compared to the control group (4-AP injected only), and the significance level was $p < 0.05$. These animals survived the experiments: the seizures symptoms decreased gradually, and the animals recovered.

2.2. Immunohistochemistry

Polyclonal c-fos antibody (#sc-52, raised in rabbit; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the peroxidase–antiperoxidase (PAP) method were used. The specificity of the primary antibody (#sc-52) was tested in our previous experiments with Western blotting (Tóth et al., 2015). The sections were pretreated with 1.5% H_2O_2 and rinsed in 0.1 M phosphate-buffered saline (PBS). Then, they were incubated in 20% normal pig serum, next, in primary c-fos antibody (1:1000 in 20% normal pig serum in PBS and 0.2% sodium azide), and then in donkey anti-rabbit IgG (1:40; Jackson Immuno-Research, West Grove, PA, USA). The secondary antibody was detected with the PAP technique (PAP complex diluted to 1:1000; Jackson Immuno-Research, West Grove, PA, USA). The peroxidase reaction was localized with diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich), yielding a brown reaction product. The sections were mounted on glass slides and covered with Entellan® (Fluka).

2.3. Morphometric analysis of the immunohistochemical data

From every animal, five sagittal plane sections of the vermis were selected. Areas of interest (AOIs) for counts of c-fos immunoreactive (c-fosIR) neuronal nuclei were from every cerebellar lobule (I–X) from the granule cell layer. The Purkinje-cell layer and the molecular layer were not investigated. These layers will be analyzed in further studies. Within each AOI, the c-fosIR cell nuclei were counted using a Nikon