The role of the cerebellum in acute generalized convulsions

Summary of Ph.D. thesis

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ABBREVIATIONS

AOI:	area of interest;
AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid;
ANOVA:	analysis of variance;
4-AP:	4-aminopyridine;
DAB:	3, 3'-diaminobenzidine tetrahydrochloride;
EDTA:	ethylenediaminetetraacetic acid;
fMRI:	functional magnetic resonance imaging
GABA:	γ-amino butyric acid;
GFAP:	glial fibrillary acidic protein;
GTCS:	generalized tonic-clonic seizure;
IgG:	immunoglobulin G;
i.p:	intraperitoneal;
MCP:	middle cerebellar peduncle;
MCPL:	middle cerebellar peduncle lesion;
MRI:	magnetic resonance imaging
mRNA:	messenger ribonucleic acid;
NMDA:	N-methyl-D-aspartic acid;
PAP:	peroxidase-anti-peroxidase;
PBS:	phosphate buffered saline;
PVDF:	polyvinylidene fluoride;
RT-PCR:	reverse transcription polymerase chain reaction;
SDS-PAGE:	sodium dodecyl sulphate polyacrylamide gel electrophoresis;
SOC:	sham-operated control.

1. INTRODUCTION

1.1.Morphological findings in epilepsy

Many brain regions and their principal neurons are involved in acute seizures and chronic epilepsy. The most important regions are the pyramidal cells of the neocortex, the relay cells thalamus, the pyramidal cells of the entorhinal cortex, parahippocampal gyrus and the hippocampus. The repeated activation of these neuronal networks causes neuropathological alterations which resemble those observed in human epilepsy. In some human epilepsies, the atrophy of the cerebellum is also detected. The histology of autopsy material reveals the shrinkage of the cerebellar cortex and the decrease of the number of Purkinje cells. The neuropathological investigations proved that the Purkinje cells are sensitive to seizures:in chronic epilepsy the number of Purkinje cells is decreased significantly, and a reactive Bergmann gliosis was observed as well. Although seizures are rarely generated primarily in the cerebellum, several epileptic patients present cerebellar symptoms and cerebellar epileptic foci have been demonstrated, too. Despite its long recognition there have been very few studies regarding the anatomical distribution of cerebellar damage in epilepsy. The neocortico-ponto-cerebellar connections often participate in these pathologies. Whether the presence of cerebellar atrophy modifies the seizure threshold remains to be clarified. The participation of the neuronal types of the cerebellar cortex in repeated seizures needs clarification too.

1.2 Glutamate transmission and glutamate receptors in the cerebellum

The cerebellar granule cell is a glutamatergic neuron, which has NMDA and AMPA receptors on its dendritic tree. The granule cell dendrites receive strong afferentation from the glutamatergic mossy fiber system. The NMDA-mediated postsynaptic effects include Ca^{++} influx and plasticity changes. The AMPA-mediated effects include desensitization because of the large size of the mossy axon terminal. The pontine nuclei provide the largest part of the mossy fiber afferents to the cerebellar cortex. In epilepsy, the increased extracellular glutamate level can cause neuronal damage and degeneration. Glutamate released from the mossy synapse obviously stimulates a large number of cerebellar granule cells. This endurance of the cerebellar glutamate transmission precipitated massive c-fos protein expression as proven by our previous experiments.

1.3 The AMPA and the NMDA receptor antagonists

The ionotropic NMDA-type glutamate receptor antagonist MK-801, ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cycloheptan-5,10-imine maleate, dizocilpine) is a selective, non-competitive antagonist, which has anticonvulsant and neuroprotective effects, too. The low-affinity NMDA receptor blocker amantadine (1-aminoadamantane) has also anticonvulsant and neuroprotective roles. The 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. Previous experiments from our laboratory proved that seizure-induced c-fos protein expression in the neocortex and hippocampus can be significantly inhibited by MK-801, amantadine (memantine) and GYKI-52466 pretreatment. Closer investigation of the different receptor blockers revealed different action mechanisms in case of the NMDA- and AMPA receptor antagonists. The aim of 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 compare their effectiveness on local neuronal activation (c-fos expression) and on the complex behavioural symptoms of the 4-AP convulsion.

1.4 The 4-aminopyridine (4-AP) model of epilepsy

In our experiments we have used 4-aminopyridine (4-AP) for seizure induction. Aminopyridines are N-heterocyclic amines, which can block the voltage-dependent K^+ channels. The increased presynaptic activity caused by 4-AP increases synaptic vesicle exocytosis. The increased synaptic activity induces an intensive c-fos expression in many brain regions. The postsynaptic *c-fos* mRNA expression correlates well with the presynaptic release of excitatory neurotransmitters. Our previous studies proved that c-fos protein is a reliable marker of neuronal hyperactivity in 4-AP induced acute seizures. During the 4-AP seizure the increase of the number of c-fosIR cell nuclei displayed a characteristic pattern: maximum numbers of the c-fosIR cells were seen at 1 hour after 4-AP treatment in the neocortex, dentate gyrus and striatum.

2. OBJECTIVES

I. The aim of our studies was to investigate and describe the timing and anatomical distribution of the activated granule cells in the cerebellum following 4-AP seizures, using c-fos immunohistochemistry and Western blotting.

II. The aim of our work was the development of a surgical method of the unilateral MCP lesion in laboratory rats.

III. We intended to clarify the role of the mossy fibers seizure-related cerebellar activation, through the unilateral lesion of the MCP by investigating the c-fos expression of the cerebellum.

IV. We wanted to investigate the role of glutamate receptors in cerebellar seizures by evaluating the changes in c-fos expression in the 4-AP seizure model following pretreatment with AMPA and NMDA receptor antagonists.

3. MATERIALS AND METHODS

3.1. Animals handling

Adult male Wistar rats weighing 200-250 g were housed in a light- and a temperature-controlled room (lights on between 6:00 a.m. and 6:00 p.m.; 23^{o}C), and had free access to food and water. The animals were kept and handled during the experiments (including surgery) in accordance with the standards and prevailing laws and ethical considerations of the European Union (European Community Council Directive; 2010/63/EU). Written permission of the protocols of the experiments was obtained from the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary. In the first group of animals (n=9) 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 in 1.0 mg/ml concentration). Control animals (n=9) received the same volume of physiological saline, i.p. Rats treated with 4-AP displayed generalized tonic-clonic motor convulsions. At the end of the convulsions, *i.e.* 1.5, 2 and 3 h following the i.p. 4-AP injection, the rats were deeply anesthetized with diethyl-ether (Fluka). The chest was opened, the ascending aorta was cannulated and the animal was perfused with cold fixative. Following transcardiac perfusion, the brains were dissected

and on sagittal plane frozen sections (25 μm thin) were cut on a freezing microtome (Reichert, USA) and used as free-floating sections in c-fos immunohistochemistry.

3.2 Pretreatment with glutamate receptor antagonists

The NMDA receptor antagonists MK-801, amantadine (Sigma, St. Louis, MO) were dissolved in saline. The AMPA receptor antagonist GYKI-52466 (Sigma, St. Louis, MO) was dissolved in 50% DMSO (DMSO: dimethyl-sulphoxide; Sigma, St. Louis, MO; . The animals were divided into six groups with four animals per group. In the first three groups the animals were pretreated with glutamate receptor antagonists MK-801 (2 mg/kg), amantadine (50 mg/kg), GYKI-52466 (50 mg/kg). After the pretreatment (15 min following the antagonist administration) the convulsant 4-AP was administered intraperitoneally (dose: 5mg/kg). In the second 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 seizure symptoms were observed and the latencies of the first generalized tonic-clonic seizure (GTCS) were measured. At the end of the observation (2 h following the 4-AP injection) the brains were dissected and c-fos immunohistochemistry was carried out.

3.3 Behavioural studies

Ten adult male Wistar rats were used in every group. The animals were pretreated with glutamate receptor antagonists MK-801 (2 mg/kg), amantadine (50 mg/kg) and GYKI-52466 (50 mg/kg) intraperitoneally. After the pretreatment (15 min later) the convulsant agent 4-AP was administered intraperitoneally (5mg/kg). In the control groups animals received the solvent of the antagonists and the 4-AP. The seizure symptoms were registered: 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

$p < 0.05$.

3.4 Morphometric analysis of the immunohistochemical data

From every animal, 5 sagittal plane sections of the vermis and the hemispheres 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. Within each AOI, the c-fosIR cell nuclei were counted using a Nikon Eclipse 600 microscope equipped with a SPOT RT Slider digital camera (1600 x 1200 dpi in 8 bits), with the help of Image Pro Plus 4.5 morphometry software (Media Cybernetics, Bethesda, MD, USA. Cell counts were done using a 10x objective, and the AOI (0.25 mm^2) included a relatively large area of the granule cell layer. Differences in the number of c-fosIR cells in the control and the convulsing animals were analysed with one way analysis of variance (ANOVA), followed by the Bonferroni *post hoc*test. A significance criterion of 0.05 was used. The statistical analysis was performed with the SPSS 9.0 software (SPSS, IBM, Chicago, IL, USA).

3.5 Western blotting

Animals (MCPL: 7 animals; SOC: 6 animals) were deeply anesthetized with diethyl-ether, decapitated and the cerebellum was removed on ice. The two hemispheres and the vermis were separated and quickly frozen in liquid nitrogen. The samples (hemispheres and vermis, separately) were homogenized in ice cold buffer containing 0.1 mM phosphate-buffered saline (PBS), 5 mM EDTA, protease inhibitors (Complete Mini, F. Hoffmann-La Roche Ltd., Switzerland), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1% Triton X-100.

SDS-PAGE was performed according to the method of Laemmli (1970) with 12% (w/v) polyacrylamide in the separating gel. Proteins were then transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). After being blocked in PBS containing 5% (w/v) low-fat milk powder, blots were exposed to antibodies against c-fos (sc-52) in 1:1,000 dilution, or beta actin (sc-47778) in 1:10,000 dilutions. Antibodies and washing steps were carried out in PBS containing 1% (w/v) low-fat milk powder and 0.1% Tween-20 detergent. Bound antibodies were detected with horseradish peroxidase labelled anti-mouse IgG (sc-2031; in 1:10,000) or anti-rabbit IgG (sc-2030; in 1:2,000), and the reaction was detected with enhanced chemiluminescence reagent as a substrate for peroxidase, and

captured on x-ray film. The film was later scanned and analysed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), other chemicals from Sigma-Aldrich (St. Louis, MO, USA). Results were analysed with one way analysis of variance (ANOVA), followed by the Bonferroni *post hoc* test.

3.6 Neurosurgery

The animals (n=15) were anesthetized with the i.p. injection of a mixture of ketamine (80 mg/kg), pentobarbital (20 mg/kg) and atropine (0.01 mg/kg). The heads were fixed in a stereotaxic instrument. The skull was opened in the retroauricular region on the left side with a dental drill. The left middle cerebellar peduncle (MCP) was exposed through the dissection of the flocculus. The MCP was destroyed with an electro-coagulator (Diatrom80; Fazzini, Italy). The skull window was closed with the bone- and muscle flaps and the skin was sutured. Sham operated control (SOC) animals (n=15) underwent craniotomy, but the dura mater was not opened. The animals survived 2 weeks following the surgery, then they were treated with 4-AP, as described above. Following the seizure experiments c-fos immunohistochemistry and cell counts were performed. Another group of the operated animals (6 MCPL and 6 SOC) was used to detect c-fos protein with Western blotting (the blotting protocol was described above). Statistical analysis on the operated animals was identical to previous descriptions.

4. RESULTS

4.1. Animal behaviour

The symptoms and the electroencephalography of the motor seizures elicited by the 4-AP injection have been described in detail. We observed similar symptoms and finally the generalized tonic-clonic seizure (GTCS) in every animal. The latency of the GTCS was 32.5 min in an average of 10 animals. The injection of 4-AP precipitated motor convulsions and GTCS. In case of MCP lesioned animals the latency of the GTCS was longer: it was 41.5 min in the average. We did not perform statistical analyses of the seizure symptoms and latencies in MCPL animals. SOC animals did not present any symptoms following the surgery.

In those animals which were pretreated with MK-801 the latency of the GTCS increased significantly. Amantadine and GYKI-52466 did not influence the latency of the GTCS. However, the number of the animals displaying GTCS decreased in every pretreated group: in the group pretreated with MK-801 only 80% of the animals, in the amantadine group only 70% of the animals and in the GYKI-52466 group only 60% of the animals displayed GTCS. Animals without GTCS displayed only shivering, minor limb clonuses and increased exploratory activity. In the control (4-AP treated) group 100% of the animals displayed GTCS.

4.2 Number of c-fos immunoreactive cell nuclei in the cerebellar vermis following 4-AP induced seizures

The GTCS caused by the 4-AP induced intensive c-fos immunostaining in the granule cell layer of the cerebellar hemispheres and vermis. The c-fos immunostaining following the 4-AP injection showed characteristic appearance in the vermal lobules. In the present study, only the granular layer has been quantified: the c-fosIR nuclei were counted according to the lobules. Control animals displayed a very small number of c-fosIR nuclei, while the 4-AP treated animals showed a large increase of the c-fos expression. The number of c-fosIR nuclei in the granular layer was raised significantly at 1.5 h and reached a maximum at 3 h. The numbers of c-fosIR nuclei were significantly higher at 1.5 h in every vermal lobule. The numbers gradually increased further at 2 h and reached a maximum at 3 h. The different lobules (I-X) were activated similarly, except lobule V, where maximum activation was seen at 2 h and the c-fosIR values decreased at 3 h.

4.3 Number of c-fos immunoreactive cells in the cerebellar vermis following pretreatment with glutamate receptor antagonists

According to our previous experiments the number of c-fos-IR cell nuclei raised significantly at 1.5 h and reached maximum at 3 h, therefore the 2 h survival time was choosen for countings in these pharmacological experiments. C-fosIR cell nuclei were counted according to every vermal lobule. In the control animals (treated only with 4-AP) the pattern and dynamics of the granule cell activation were similar to that previously observed and described. A large number of c-fos positive cells were detected in every vermal lobule in the granular layer of the cerebellar cortex 2 h following the 4-AP injection. This number of the c-fosIR cell was significantly greater than that in non-

convulsing control animals. Pretreatment with GYKI-52466, MK-801 and amantadine resulted in a significant decrease of the number of c-fosIR positive granule cells compared to animals treated only with 4-AP. Decreases of the c-fosIR granule cell numbers were similar in the anterior and posterior lobes and in every vermal lobule. The summated counts of the entire vermis also displayed significant differences: decreases of the c-fosIR cell nuclei in the animals pretreated with the NMDA- and AMPA antagonists were highly significant.

4.4 C-fos immunoreactivity and Western blotting after transection of the middle cerebellar peduncle

In order to examine the effect of the MCPL we used the immunohistochemical analysis of c-fos expression of the cerebellar granule cells in MCPL and SOC animals. The animals were injected with 4-AP and 2 h after the injections quantitative immunohistochemistry was performed (see Methods). The number of c-fosIR nuclei of SOC animals did not differ from that seen in intact, non-operated animals. Quantitative examination proved that the c-fos expression of granule cells decreased significantly in the operated hemisphere of MCPL animals compared to the SOC animals and to the non-operated hemisphere of the MCPL animals. We also tested the effect of the MCPL on the c-fos protein content with Western blotting of cerebellar tissue from control and convulsing animals. The results showed that the c-fos protein levels decreased significantly in the vermis and in both hemispheres (in the operated and in the contralateral hemispheres) of the MCPL animals, compared to those of the SOC animals ($p<0.001$). The decrease in the operated hemisphere was larger than that in the contralateral one. No significant differences in the c-fos contents were detected between the operated and non-operated (contralateral) hemispheres in the MCPL animal, although the operated side contained a slightly weaker c-fos signal.

4.5 Histology and immunohistochemistry cerebellar cortex after the transection of the middle cerebellar peduncle

Thin coronal sections of the cerebellum and brainstem revealed that as the result of neurosurgery, the MCP was completely destroyed. On the side of the surgery, the flocculus

was also absent. No other neuropathological damages and no signs of inflammation were observed in the surgery location. The normal cerebellar cortex displays strong synapsin I immunoreactivity in the granular layer. On the side of the MCPL synapsin I immunoreactivity disappeared from the granular layer of the cerebellar cortex. The densitometry measurements of the cerebellar layers strongly supported this light microscopic observation. The contralateral cerebellar cortex of the MCPL animals, and the cerebellar cortex of SOC animals displayed normal synapsin I staining. The GFAP immunoreactivity was strong on the operated side: the radial glia and scattered astrocytes contained large amounts of GFAP in every cerebellar cortical layer. The contralateral MCPL and SOC cerebellar cortices contained much less immunostaining. No densitometry evaluation of the GFAP staining was performed.

5. DISCUSSION

5.1.C-fos expression is a reliable marker of neuronal hyperactivity

Seizures induced by chemical convulsants lead to a rapid, massive and transient induction of *c-fos* mRNA and protein in several brain regions. The postsynaptic *c-fos* mRNA expression correlates well with the presynaptic release of excitatory neurotransmitters , and the detection of the c-fos protein is therefore suitable for the histological mapping of neuronal hyperactivity. Our previous studies proved that c-fos protein is a reliable marker of neuronal hyperactivity in 4-AP induced acute seizures. During the 4-AP seizure the increase of the number of c-fosIR cell nuclei displayed a characteristic pattern: maximum numbers of the c-fosIR cells were seen at 1 hour after 4-AP treatment in the neocortex, dentate gyrus and striatum.

5.2 C-fos expression pattern of the cerebellar granule cells following 4-AP induced seizures

The effects of 4-AP-induced seizure were similar in the cerebellar cortex: strong c-fos protein expression was detected in the granular layer. Important observation of the present experiments was that the activation of the cerebellar granule cells lasted significantly longer than the activation of the hippocampal granule cells, the activation of neurons of the striatum and the activation of the neurons of the neocortex. The NMDA-mediated

postsynaptic effects include Ca^{++} influx and plasticity changes. The AMPA effects include desensitization because of the large size of the mossy axon terminal.

The number of the granule cells is approximately four millions in one mm^3 cerebellar cortex tissues. One mossy fiber glomerulus contacts at least 50 granule cell dendrites. Considering that a single granule cell has 4-5 dendrites and a mossy axon gives at least 20-25 axonal glomeruli, this means several hundreds of granule cells will be activated for every mossy axon. This is a strong divergence at the first cerebellocortical synapse, which is not seen in other circuits. On the other hand, the mossy axon terminal contains a large number of synaptic vesicles: the large synaptic vesicle population possibly results in a sustained strong excitation because of the large amount of released glutamate, and also the spill over of glutamate around the glomerulus. This divergence on the level of the first synapse is possibly responsible for the slow increase of the number of the c-fosIR cell nuclei in the cerebellar cortex during the 4-AP seizure. The presence of the NMDA receptors on the granule cell dendrites possibly helps the effectivity of the mossy fiber stimulation and the *c-fos* expression. The different vermal lobules showed very similar activation dynamics: the number of the c-fosIR cell nuclei increased stepwise from 1.5 h to 3 h.

5.3 Effects of the glutamate receptor antagonists on the seizure symptoms and the c-fos expression in the cerebellar cortex

The pontine nuclei provide the largest part of the mossy fiber input to the cerebellar cortex. The glutamatergic pontocerebellar system terminates in form of mossy fibers in the granular layer of the cerebellar cortex stimulating mainly granule cells and Golgi cells. Whilst these mossy fibers use mainly glutamate, granule cells possess NMDA- and AMPA receptors on their dendrites. Both NMDA- and AMPA receptors are able to induce the expression of transcription factors through the strong postsynaptic Ca^{++} influx. We think that 4-AP in the cerebellum induced c-fos expression partly through the increased release of glutamate from the mossy fibers, and as a consequence the increase of Ca^{++} influx into the postsynaptic cells. Cerebellar granule cells possess Ca^{++} permeable AMPA receptors which means, that Ca^{++} entry will be facilitated at postsynaptic structures surrounding the mossy fibers. This ionotropic glutamate transmission mechanism explains our observations on the significant increase of c-fos protein in the cerebellar cortex in 4-AP seizures. We think that our presented experiments also support this postulated mechnism:

the pharmacological antagonism of the NMDA- and AMPA receptors decreased the number of the c-fosIR cells significantly. We conclude that these drugs can decrease the cerebellar activity through postsynaptic mechanisms, acting at the dendrites of the granule cells and decreasing their firing upon the mossy fiber activity. The pharmacologically inhibited firing of the granule cells is reflected by the decrease of the postsynaptic c-fos protein expression in the granular layer of the cerebellar cortex. It has been reported that the density of the AMPA receptors is very high in the cerebellar cortex. The granule cells and the Golgi cells possess AMPA receptors. The GluR1 subunit is also expressed by Bergmann glia cells. Glial AMPA receptors play important role in glutamatergic transmission of the cerebellar cortex. As to the pharmacological properties of cerebellar glial AMPA receptors, it was proven, that GYKI-52466 was an effective antagonist in Bergmann glia-mediated aspartate release. The MK-801 is a high-affinity NMDA (N-methyl-D-aspartate) receptor antagonist. Amantadine is a low affinity open-channel blocker of NMDA glutamate receptors. These drugs have potential neuroprotection effect as well. The NMDA receptors obviously play an important role in the fast synaptic transmission of the cerebellar mossy fiber synapses. There are literature data, that presynaptic NMDA receptors containing NR2D subunit regulate GABA release in the cerebellar cortex. The antagonists acting at these NMDA receptors (*e.g.:* ifenprodil) may have inhibitory effect on GABA release, further complicating the pharmacological effects of glutamate in the cerebellar cortex.

5.4 The role of the mossy fibers in seizure induced c-fos expression of the cerebellar granule cells

The role of mossy fiber afferents in *c-fos* gene activation of the granule cells was clearly supported by our experiments: deafferentation through the transection of the MCP caused an overall, significant decrease of detectable c-fos protein in the cerebellar cortex. The unilateral cutting of the MCP decreased the seizure induced c-fos protein expression not only on the operated side but also in the vermis and in the contralateral, non-operated side, as detected by the Western blots. This is in accord with the characteristic branching pattern of the mossy axons entering through the MCP: the axons give branches, which innervate the ipsilateral cortex, the vermis, then pass to the contralateral cerebellar cortex through the cerebellar commissure. Our results clearly proved that following the destruction of the MCP, the c-fos immunoreactivity of the granular layer decreased significantly in both

cerebellar hemispheres. This large scale activation involved the entire posterior lobe (the main target of the pontocerebellar axons. Due to this synchronized afferent activation, the cerebellar cortex was unable to participate in movement regulation – instead, the strong granule cell excitation generated a failure of the cerebellar output mechanisms: thereby increasing the breakdown of motor regulation in epilepsy. We hypothesize, that the strong mossy fiber activation is partly due to the local effect of 4-AP: it is also caused by the strong excitation of the corticopontine tracts. The forebrain seizure is generated by neocortical-entorhinal-hippocampal circuits. This forebrain activity will spread to the pontine nuclei and the reticulotegmental nucleus. The mossy fibers originating from these pontine structures will be activated and cause a long-lasting and large-scale excitation of the cerebellar cortex. This excitation is reflected by the significant increase of the number of c-fosIR cell nuclei. If the cerebellar activation is long-lasting and recurrent (as it can be in chronic epilepsies), it might be responsible for the cerebellar atrophy seen regularly in autopsy of patients having had chronic epilepsy. However, as seen from our immunohistochemical and Western blotting results, the activating power of the mossy axon is larger on the side of the corresponding MCP. The Western blots showed a significant decrease on the contralateral hemisphere, and another, larger decrease on the operated side.

6. CONCLUSIONS

1. Generalized tonic-clonic seizures precipitated by the potassium channel blocker 4-aminopyridine, caused a long-lasting c-fos protein expression in the granular layer of the cerebellar cortex, indicating long lasting neuronal hyperactivity.
2. The vermal lobules (I-X) displayed a uniform activation pattern pointing to the concerted activation of cerebellar afferents during the seizure.
3. Transection of the middle cerebellar peduncle on the left side abolished most of the c-fos immunoreactivity, proving the pivotal role of the mossy fiber afferents in cerebellar seizures.
4. The importance of the NMDA and AMPA-type glutamate receptors in the precipitation and maintenance of cerebellar seizures have been proven by the significant decrease of seizure-induced c-fos immunoreactivity of the granular layer following the administration of receptor antagonists.

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