Research report

Repeated 4-aminopyridine seizures reduce parvalbumin content in the medial mammillary nucleus of the rat brain

Sándor Vizi, Andrea Bagosi, Beáta Krisztin-Péva, Károly Gulya, András Mihały,*

*Department of Zoology and Cell Biology, University of Szeged, Szeged, Hungary
bDepartment of Anatomy, Histology and Embryology, University of Szeged, P.O.B. 427, H-6701 Szeged, Hungary

Accepted 31 August 2004
Available online 3 October 2004

Abstract

Parvalbumin (Pv) containing fast spiking neurons play a crucial role in synchronizing the activity of excitatory neuronal circuits in the brain. Alterations of parvalbumin content in these neurons can affect their spike characteristics and, ultimately, may increase the susceptibility of neuronal circuits to epileptic seizures. In the present study, we examined whether repeated 4-aminopyridine (4-AP)-induced seizures modify the regional parvalbumin contents in the rat brain. 4-Aminopyridine was injected intraperitoneally in adult rats, controls received the solvent. Animals were sacrificed at 3 h after a single acute treatment, or following repeated, daily treatments of 12 days. In situ hybridization (ISH) indicated significantly decreased parvalbumin mRNA level in the medial mammillary nucleus (MM) at 12 days. Western blotting revealed 20.1% significant decrease of parvalbumin content in the medial mammillary area, while parvalbumin immunohistochemistry indicated no change of the number of immunoreactive cells in the medial mammillary nucleus. The results reveal the downregulation of the transcription of the parvalbumin gene and the decrease of parvalbumin synthesis in medial mammillary nucleus neurons in response to experimental seizures.

1. Introduction

Epilepsy comprises heterogeneous chronic disorders of the nervous system characterized by spontaneous recurrent seizures [51]. Seizure activity itself is due to abnormally synchronized rhythmic activity of large assemblies of neurons [32]. The complex pathological mechanisms responsible for evoking epileptiform neuronal discharges are intensively investigated at the molecular, cellular and system levels of the nervous system.

Current molecular epilepsy research employing both forward and reverse genetics approaches has revealed more than 60 candidate genes linked to different forms of epileptic syndromes [39]. Intriguingly, the affected genes code not only for molecules playing a direct role in shaping neuronal activity, but also for other proteins involved in such diverse cellular processes as intracellular signalling, regulation of gene expression, cell division, cell migration and cell death. The ongoing functional characterization of these genes may reveal crucial steps in seizure generation at the molecular level. Even then, full appreciation of the interacting processes leading to epileptic seizure generation can only be achieved by incorporating results from cellular and system studies into experimental epilepsy models.

To date, various cellular processes have been implicated in seizure generation and, accordingly, different hypotheses have been proposed [12,52–54]. Several epilepsy models assign a crucial role for specific interneuron classes in...
seizure generation [51] since the synchronization of the activity of cortical excitatory neurons and, thus, generation of rhythmic discharges in cortical networks is largely dependent on these cells. In turn, seizure-inducing alterations in the physiological activity of interneurons have also been attributed to changes in the activity pattern of their afferent excitatory input [8].

Parvalbumin (Pv), a highly diffusible cytosolic Ca\(^{2+}\)-binding protein [24], is a cellular marker for fast spiking interneurons in various regions of the rat brain [25–27]. The discharge characteristics of these interneurons are supposed to be critically dependent on the Ca\(^{2+}\)-buffering action of Pv [22,24,29,30,33,47]. In the present study, we applied the convulsant agent 4-aminopyridine (4-AP) [44] to induce generalized seizures. Then, we examined whether the experimentally induced single and repeated seizures modified the Pv content in various regions of the rat brain. Post-seizure modification of the Pv content in fast-spiking neurons could be both indicative of, and a reason for the alterations in their physiological function.

2. Materials and methods

2.1. Experimental animals and treatments

Male Sprague–Dawley rats (120–140 g) were housed in groups under standard conditions (temperature 23 °C, lights on from 06:00 to 18:00 h) and with free access to water and food. The animals were injected intraperitoneally with 0.067% (w/v) solution of the convulsant 4-AP (Sigma; St. Louis, MO, USA) dissolved in physiological saline (0.9% NaCl in distilled water). The applied dose was 5 mg/kg body weight, as this concentration proved to be convulsive in previous studies [34,35]. Control animals received appropriate volumes of physiological saline. In acute experiments, 4-AP treated and control rats received a single injection, and after 3 h were sacrificed under deep diethyl ether anesthesia. In chronic experiments, animals were injected either with 4-AP or with saline daily for 12 days, and sacrificed 3 h after the last injection, as described above. The animals were observed for 2 h after every injection, and their convulsive behaviour was rated according to Racine [45]. Animals were killed in groups of five for Pv in situ hybridization (ISH), and in groups of four for Western blotting and in groups of three for Pv immunohistochemistry (IHC).

For ISH, brains were quickly removed, embedded in Cryomatrix (Shandon Scientific; Pittsburgh, PA), immediately frozen at −70 °C, and stored at −20 °C until further processing. Frozen brains both from the acute and chronic experiments were sectioned on a cryostat in the coronal plane at the thickness of 15 μm. Sections cut at bregma −3.14 and −4.52 [41] were thaw-mounted onto 3-amino-propyl-triethoxysilane (Sigma) coated slides, air-dried, and stored at −20 °C until further processing.

For IHC, animals were deeply anesthetized with diethyl ether and perfused transcardially with 200 ml of 0.1 M phosphate buffered saline (PBS, pH 7.4), followed by 300 ml of 4% phosphate buffered paraformaldehyde (pH 7.4). The brains were removed and postfixed in 4% phosphate buffered paraformaldehyde (pH 7.4) for 1 h. After postfixation, the brains were cryoprotected overnight in 30% sucrose in 0.1 M phosphate buffer (pH 7.4). Coronal frozen sections were cut on a freezing microtome at the thickness of 24 μm at bregma −4.52 and processed for IHC.

All procedures of the animal experiments were licensed by the Committee for Animals in Experimental Research, University of Szeged, according to the directive of the European Council (86/609/EEC) and to the Hungarian Animal Act.

2.2. cRNA probes

The only Pv gene in the rat [6] contains five exons and codes for two mRNA species by means of alternative polyadenylation [17]. A template for a gene-specific cRNA probe was created by isolating the 133 bp long third exon of the rat Pv gene [3] by genomic polymerase chain reaction (PCR) using the primers 5′-CCCCCAACAGCTGCA-GACTCC-3′ and 5′-TTTCCCCAGCTCATCCTCCT-CAATG-3′. The PCR product was inserted into the pcDNA3 vector (Invitrogen, Carlsbad, CA) between BamHI and EcoRI restriction sites and cloned using standard methods [46]. Identity of the insert was confirmed by sequencing (AB 373 DNA Sequencer; PE Applied Biosystems, Foster City, CA). For the production of antisense and sense Pv cRNA probes, the vector construct was linearized either with BamHI or EcoRI (Promega, Madison, WI, USA), respectively. Then, the linearized plasmid was transcribed in vitro by using Riboprobe System-T7 and Riboprobe System-SP6 (Promega) according to the manufacturer’s instructions. For radiolabelling, [35S]UTPαS (ICN Biomedicals; Costa Mesa, CA) was incorporated during the syntheses of the cRNA probes. The antisense and sense Pv [35S]cRNA probes were purified by size exclusion chromatography using ProbeQuant G-50 Sephadex microcolumns (Pharmacia Biotech; Uppsala, Sweden) and their specific activities were determined to be 3.1×10⁷–6.4×10⁷ cpm/pmol by liquid scintillation counting.

2.3. In situ hybridization

Our protocol for in situ hybridization has been previously described in detail [56,57]. Briefly, coronal rat brain sections were fixed for 5 min in 2× SSC containing 4% formaldehyde at RT and washed twice in 2× SSC for 1 min at RT. Then, the sections were rinsed in 0.1 M triethanolamine containing 0.25% acetic anhydride and 0.9% NaCl, pH 8.0 for 5 min at RT, dehydrated, and air-dried. Next, the sections were hybridized in 50 μl hybridization solution (50% formamide, 6× SSPE, 5× Denhardt’s reagent, 10% dextran sulfate, 50 mM DTT, 100 μg/ml salmon sperm.
DNA, and 50 μg/ml yeast tRNA containing a $^{35}$S-cRNA probe. The concentrations of the Pv antisense and sense hybridization solutions ranged from 247 to 288 fmol/ml. Hybridization was performed under Parafilm coverslips in a humid chamber at 55 °C for 24±0.5 h. The sections were rinsed once in 2× SSC/50% formamide at RT for 5 min, twice in 2× SSC/50% formamide at 50 °C for 10 min, and then in 2× SSC at RT for 5 min. The sections were next incubated in 1× TE containing 0.5 M NaCl and $1.32 \times 10^{-3}$ M Kunitz U (25 mg protein)/ml RNase A at 37 °C for 30 min, and rinsed in 2× SSC/50% formamide at 50 °C for 10 min and in 2× SSC at 50 °C for 10 min. Sections were dehydrated, air-dried and processed for phosphorimaging.

2.4. Western blotting

Rats in diethyl-ether anesthesia were decapitated 3 h after the last 4-AP or saline injection. The heads were immersed in liquid nitrogen, and the brains were dissected on ice. The mammillary area was cut out, chilled in liquid nitrogen and stored at −80 °C until use. The number of animals was 16: 8 treated with 4-AP for 12 days, and 8 controls (injected daily with saline for 12 days). The tissues were homogenized in 50 mM Tris–HCl (pH 7.5) containing 2 mM phenylmethanesulfonyl fluoride (PMSF), 150 mM NaCl, 0.1% Nonidet-P-40, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 2 mM EDTA. Protein concentrations were determined according to the method of Lowry et al. [31]. Thirty-five micrograms protein was separated on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Membranes were blocked for 1 h in 5% nonfat dry milk in Tris–HCl (pH 7.5) containing 0.9% NaCl (TBS) and 0.05% Tween 20, and incubated overnight with mouse anti-Pv antibody (Sigma PA-235; dilution 1:3000). After 5×5 min washes in TBS-Tween 20 on room temperature, the membranes were incubated for 1 h with biotinylated goat anti-mouse IgG (Vector Laboratories; Burlingame, CA, USA; dilution 1:1000), and washed three times as before. The enhanced chemiluminescence method (ECL Plus Western blotting detection reagents; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was then used to reveal immunoreactive bands, according to the manufacturer’s protocol. The other chemicals were from Sigma. The intensity of the bands was quantified by densitometry and expressed as pixel volume (pixel intensity×area) using the Gel-Doc system (Biorad). For quantitative comparisons, each value was calculated based on at least seven independent determinations. An analysis of variance (ANOVA) followed by post hoc analysis (Tukey’s multiple comparison) was used to evaluate the significance.

2.5. Immunohistochemistry

Coronal, frozen brain sections (24 μm thin) were reacted with monoclonal mouse anti-Pv antibody (Sigma PA-235, dilution 1:15,000). For the detection of the primary antibody, biotinylated goat anti-mouse IgG (Vector) and streptavidin-HRP (Vector) were employed. The immunoperoxidase reaction was developed by using 3′,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) as a chromogen. The sections were mounted on microscope slides, dehydrated and coverslipped with Entellan (Fluka). Details of the immunohistochemical procedure are found in Ref. [35].

2.6. Imaging and image analysis

Hybridized rat brain sections and membrane standard scales [40], previously calibrated to brain paste standard scales [57], were co-exposed to SR Cyclone storage phosphor screens (Packard Instrument, Meriden, CT) for 24 h at RT. Linearity of the densitometric graph of the detection system was confirmed previously [56]. Phosphorimages were captured with Cyclone Storage Phosphor System (Packard) at 600×600 dpi resolution and analysed by the computer program OptiQuant version 4.0 (Packard). Images of brain areas were outlined on the computer screen and their signal intensities were expressed in digital light units (dlu) per mm$^2$. Labelling values of brain areas were corrected for the screen background (less than 7000 dlu/mm$^2$) and the resulting values were expressed in net dlu/mm$^2$.

Consecutive steps of conversion of net dlu/mm$^2$ values to net ISH copy no./mm$^2$ values were described earlier in detail [56]. Labelling values of brain areas (net dlu/mm$^2$) were converted to membrane labelling values (net dlu/mm$^2$) using a previous calibration [57]. Further conversion steps can be summarized in the equation

$$RES = \frac{(LAB \times AVO)}{(LRF \times RDF \times SPA)}$$

where RES is the result (ISH copy number/mm$^2$), LAB is the labelling intensity (net dlu/mm$^2$), AVO is the Avogadro number (6.02252×10$^{23}$ copy number/mol), LRF is the labelling vs. radioactivity factor [(net dlu/mm$^2$)/(cpm/mm$^2$)], RDF is the radioactive decay factor, and SPA is the specific activity of the cRNA probe (cpm/pmol).

Correction for the tissue background was performed by taking into account the specific activities of the cRNA probes. The screen background-corrected labelling value of a brain region hybridized with the antisense Pv cRNA probe was converted to a RES value and then corrected for the average of the RES values of the co-exposed brain sections hybridized with a similar concentration of the sense Pv cRNA probe. The resulting net RES value (ISH copy number/mm$^2$) was regarded as an estimate of the regional copy no/mm$^2$ value of the Pv mRNA.

The immunoreactive cells in the mammillary complex were counted using a Nikon Eclipse 600 microscope equipped with a Spot RT Slider digital camera (1600×1200 dpi in 8 bits), and the Image Pro Plus 4.5 morphometry software (Media Cybernetics, Silver Spring, MD, USA).
The analysis was performed on five frozen sections from every animal. The areas of interest (AOI) for cell counts were selected from the medial mammillary nucleus. Following background subtraction, the threshold was adjusted so that the counting program could equally recognize pale- and deep-stained cells. Cell counts were done using a 40× objective. The AOI was the rectangular image-capturing field of the camera (222 μm×296 μm). We also measured the thickness of the section and the diameter of the cells using differential interference contrast (DIC) optics, with the Marzhauser MultiControl 2000 motorized stage. The thickness data were rectified for the refractive index of the dry objective [21]. The neuron number was obtained and corrected with the help of the following formula:

\[
\text{Cell number} = \frac{\text{Number of counted cells} \times \text{Section thickness}}{(\text{Section thickness} + \text{Cell diameter})} [1,14,20].
\]

2.7. Data processing

Data reduction was accomplished with the computer software Excel 2002 (Microsoft, Redmont, WA). For Pv ISH data, one or two outlier data points were excluded from the data sets when they compromised the normal distribution of the experimental data (examined by Shapiro-Wilk W and skewness tests, \(p < 0.05\)). Analysis of significance was carried out with independent samples Student’s \(t\)-test (\(p < 0.01\) or 0.001), ANOVA followed by a post hoc Bonferroni test (\(p < 0.05\)), and Median test (\(p < 0.05\)). For Pv IHC data, two-tailed Student’s \(t\)-test (\(p < 0.05\)) was employed. Cell numbers of Pv immuno-histochemistry measurements in the medial mammillary nucleus were analysed with independent samples \(t\)-test. A significance criterion of \(p < 0.05\) was used. The statistical analysis was performed with the SPSS 9.0 software.

Fig. 1. Seizure symptoms according to Racine [45], observed in one of the experimental animals during the 12 days of the 4-AP treatment. Stage 1: mouth and facial movements; stage 2: head nodding; stage 3: forelimb clonus; stage 4: rearing; stage 5: full motor seizure (rearing and falling with loss of postural control). Similar observations were made in every 4-AP treated animal.

Fig. 2. Expression of Pv mRNAs in the rat brain following acute (3 h; A–D) and chronic (12 days; E–H) 4-AP treatment. Coronal sections cut at bregma −3.14 and at bregma −4.52 were hybridized with antisense (A–H) and sense (I) Pv [\(^{35}\)S]cRNA probes. Arrowheads indicate the medial mammillary nucleus displaying significant changes in Pv mRNA contents in 4-AP treated animals when compared to the corresponding controls (\(p < 0.05\); ANOVA and post hoc Bonferroni tests; see main text). Arrow on B points to the reticular thalamic nucleus. Abbreviations: CA1 and CA3, CA1 and CA3 pyramidal cell layer of the hippocampus; DG, granular cell layer of the hippocampus; Fr, frontal cortex; MolHC, molecular layer of the hippocampus; Oc2L and Oc2M, occipital cortex 2L and 2M; RSG, retrosplenial granular cortex. Bar represents 5 mm.
3. Results

3.1. Seizure behaviour following 4-AP treatment

The administration of 5 mg/kg 4-AP caused characteristic seizure symptoms: mouth and facial movements (stage 1), head nodding and muscle tremor (stage 2), forelimb clonus (stage 3), rearing (stage 4) and generalized tonic-clonic seizure (GTCS, stage 5), with complete loss of postural control [45]. The average latency of GTCS was 30.3 min (+1.4 min). These symptoms were detected everyday, during the 12 days’ treatment, although the severity and the stages showed slight variations (Fig. 1).

3.2. Quantification of regional Pv mRNA contents in the rat brain following acute and chronic 4-AP treatment

Regional quantities of Pv mRNAs in the brains of rats subjected to acute or chronic 4-AP treatment were assessed by ISH employing Pv [35S]cRNA probes. Autoradiograms of sections hybridized with antisense Pv [35S]cRNA probes displayed a characteristic labelling pattern specific for Pv mRNAs (Fig. 2A–H). Images of sections hybridized with sense Pv [35S]cRNAs had very low level of labelling and did not exhibit brain structure specific signals (Fig. 2). Normal distribution of experimental data was confirmed for each data set except that of group 12 days 4-AP medial mammillary nucleus. In this group, several data points with very similar values compromised the normal distribution of data (skewness) even when the number of measurements was doubled by quantifying contralateral as well. The subnuclei [3] of the medial mammillary nucleus (MM) could not be separated on the ISH images (Fig. 2).

The Pv mRNA content of the forebrain regions of the 4-AP treated rats were compared to that of the corresponding control rats by using two-tailed Student’s t-test (p<0.01). None of the investigated neocortical and allocortical areas displayed changes in Pv mRNA levels in our experiments (Table 1). Daily convulsions induced by 4-AP through 12 days did not have any impact on the transcription of the Pv gene in the cerebral cortex (Table 1). In the diencephalon, Pv mRNA levels displayed a slight increase in group 3 h in the thalamic reticular nucleus (nRt) (116%), while in group 12 days significant decreases (77%, n=20, p<0.001) were observed in the MM of the hypothalamus. Data sets were also compared with ANOVA followed by post hoc Bonferroni test (p<0.05, Table 1). When regional Pv mRNA abundances in 4-AP groups were compared to those in the corresponding controls groups, ANOVA and Bonferroni tests indicated that changes of the nRt of the thalamus are not significant (Table 1). Data sets of MM were further analysed with the nonparametric Median test, which reinforced that the decrease of Pv mRNA level in group 12 days 4-AP in the MM was highly significant, when compared to 12 days control value, confirming the results for this brain region obtained with the above parametric tests (Table 1). The decreased level of Pv mRNA in the MM was visible on the in situ hybridization images, too (Fig. 2). Therefore, we further investigated the Pv content of the MM with immunological methods.

3.3. Western blotting of the mammillary area

Pv immunoreactive bands were sharp (Fig. 3). The samples from control and 4-AP-treated rats displayed a visible density difference (Fig. 3). Densitometry of the membranes revealed a 20.1±5.5% decrease of Pv signal intensity in rat MM treated with 4-AP for 12 days, compared to the controls (data from eight samples of convulsing brains and seven samples from controls). The

<table>
<thead>
<tr>
<th>Area</th>
<th>3 h</th>
<th>12 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal</td>
<td>91.1±16.4</td>
<td>102.8±19.0</td>
</tr>
<tr>
<td>Hindlimb</td>
<td>89.8±11.9</td>
<td>110.9±32.9</td>
</tr>
<tr>
<td>Parietal</td>
<td>124.8±18.4</td>
<td>125.5±17.8</td>
</tr>
<tr>
<td>Parietal</td>
<td>120.7±13.7</td>
<td>140.0±32.9</td>
</tr>
<tr>
<td>Occipital</td>
<td>155.3±14.8</td>
<td>181.3±27.9</td>
</tr>
<tr>
<td>Occipital</td>
<td>166.2±21.5</td>
<td>194.3±38.1</td>
</tr>
<tr>
<td>Temporal</td>
<td>117.1±16.8</td>
<td>147.8±21.2</td>
</tr>
<tr>
<td>Occipital 2M</td>
<td>120.9±14.4</td>
<td>143.7±16.4</td>
</tr>
<tr>
<td>Occipital 2L</td>
<td>101.3±19.1</td>
<td>108.6±16.0</td>
</tr>
<tr>
<td>Temporal 1</td>
<td>86.7±4.9</td>
<td>117.1±17.7</td>
</tr>
<tr>
<td>Temporal 2L</td>
<td>136.4±20.1</td>
<td>134.5±23.3</td>
</tr>
<tr>
<td>Temporal 2L</td>
<td>129.4±20.1</td>
<td>147.4±32.8</td>
</tr>
<tr>
<td>Temporal 1</td>
<td>165.0±13.7</td>
<td>162.0±21.6</td>
</tr>
<tr>
<td>Temporal 2M</td>
<td>152.3±6.3</td>
<td>166.9±27.1</td>
</tr>
<tr>
<td>CA1 pyramidal cell layer</td>
<td>142.4±28.9</td>
<td>142.6±32.4</td>
</tr>
<tr>
<td>hippocampus</td>
<td>122.7±38.8</td>
<td>163.1±39.9</td>
</tr>
<tr>
<td>CA3 pyramidal cell layer</td>
<td>131.7±37.8</td>
<td>184.4±65.4</td>
</tr>
<tr>
<td>hippocampus</td>
<td>149.0±40.9</td>
<td>165.8±56.0</td>
</tr>
<tr>
<td>Granule cell layer</td>
<td>120.7±22.0</td>
<td>119.6±16.3</td>
</tr>
<tr>
<td>dentate gyrus</td>
<td>100.3±17.4</td>
<td>135.8±39.7</td>
</tr>
<tr>
<td>Medial mammillary</td>
<td>766.6±93.4</td>
<td>668.0±125.8</td>
</tr>
<tr>
<td>nucleus</td>
<td>682.3±109.2</td>
<td>514.5±144.5</td>
</tr>
<tr>
<td>Lateral mammillary</td>
<td>315.3±86.6</td>
<td>267.1±78.7</td>
</tr>
<tr>
<td>nucleus</td>
<td>324.6±99.1</td>
<td>344.0±108.6</td>
</tr>
<tr>
<td>Reticular thalamic</td>
<td>862.7±113.1</td>
<td>929.3±86.5</td>
</tr>
<tr>
<td>nucleus</td>
<td>998.6±92.2</td>
<td>953.5±128.8</td>
</tr>
</tbody>
</table>

*Coronal cryostat sections cut at different levels of the rat brain were hybridized with Pv [35S]cRNA probes and then exposed to a phosphor screen. Digital images of brain sections were captured with a phosphor imager and labeling values for selected brain areas were measured in dlu/mm² units. Tissue background correction was performed by taking into account the number of the labeled nucleotides in the antisense and sense Pv [35S]cRNA probes and their radioactive decay. The presented values are ISH copy numbers (×10³)±S.D. corresponding to 1 mm² area of a 15-µm thick section. For each brain region, values for the control and 4-AP treated groups are presented in the first and second row of data, accordingly. Downward arrow symbolizes significantly decreased values for 4-AP treated animals, respectively, as compared to the corresponding control value (p<0.05; ANOVA and post hoc Bonferroni tests; see main text).
difference between control and 4-AP-treated values was significant ($p < 0.05$).

3.4. Quantitative analysis of Pv immunoreactive cells in the medial mammillary nucleus of the rat brain after 4-AP treatment

Pv immunohistochemistry was performed on brain sections from rats subjected 12-day 4-AP treatment and the control animals. The Pv immunoreactivity in the MM (Fig. 4) was similar to that described [13]. Medium-sized neurons with few dendritic processes contained Pv-like staining. Regional densities of immunolabelled cell bodies were determined for each experimental group. Values from 4-AP treated groups were compared to those from corresponding control groups (independent samples Student’s $t$-test, $p < 0.05$). The number of PV-immunoreactive neurons did not change in 4-AP-treated animals, compared to the controls (Fig. 4), although the intensity of the staining did show a visible decrease. No pathological cell forms were detected in the 12 days 4-AP samples.

4. Discussion

The analysis of the behavioural symptoms of daily 4-AP treatment on the Racine’s scale [45] proved that generalized seizures were precipitated every day during the treatment. Literature data indicate that the observed behavioural symptoms correspond to two to three short, tonic-clonic EEG convulsions, which can be detected in the neocortex, hippocampus, striatum and thalamus [18,36]. Although these seizures are not returning spontaneously, they closely resemble the EEG and cellular electrophysiological features.
of human epilepsy [18,36]. Therefore, we think that this model is suitable for the study of the role and alterations of the limbic system during repetitive, short seizures.

The main finding of our experiments is that neocortical and allocortical structures did not display changes in Pv gene transcription during repetitive, short, chemically induced convulsions. Our preliminary investigations indicate that 12-day 4-AP treatment does not induce cell loss and neuronal degeneration, or breakdown of the blood–brain barrier. Literature data, concerning the changes of Pv immunoreactive cells in experimental epilepsies relate the changes to the seizure-induced cell loss and reorganization of the local neuronal circuits [48,52]. Therefore, our results do not contradict to existing literature evidence on Pv expression in seizures.

However, the 4-AP evoked experimental seizures induced significant changes in Pv mRNA levels in the diencephalon. The nRt displayed the highest Pv mRNA density and exhibited a modestly increased Pv mRNA level 3 h after the acute 4-AP treatment. However, these mRNA changes were not significant. The nRt is known to play a crucial role in controlling the activity of thalamocortical circuits [55]. GABAergic neurons of the nRt receive excitatory input via corticothalamic axon collaterals, while they innervate and control the activity of thalamic relay neurons projecting to neocortical areas [19]. Interestingly, no elevation of Pv mRNA level in nRt was observed following 12-day 4-AP treatment.

The other important finding of our experiments is that the MM exhibited a highly significant loss of Pv mRNAs after the chronic 4-AP treatment. As no decreased cell number were observed in the mammillary nuclei in the 12 days groups, the reduction in the Pv mRNA density is likely to indicate the downregulation of the cellular mRNA content in MM. To examine whether the lower Pv mRNA abundance led to a decreased Pv protein content in MM neurons, Western blotting experiments were also quantified because Ca2+ fluxes are not regulated properly [11]. The results obtained on Purkinje cells of Pv knockout mice indicated that the absence of Pv might cause cumulative facilitation, which is a common autopsy finding in long standing human epilepsies [38]. These data indicate that hippocampal epileptic seizures can selectively activate mammillary neurons. The convulsive agent 4-AP is a blocker of voltage sensitive K+ channels [2], and is known to induce Glu and GABA release in the hippocampus accompanied by epileptiform EEG waves [18,35,42,43]. Therefore, the observed reduction of Pv content in MM following 12 days of 4-AP induced experimental epilepsy could well be due to the repetitive burst activity of the hippocampal projections to the mammillary nuclei [4].

A similar phenomenon has been described in the hippocampus of epilepsy prone Mongolian gerbils, where Pv immunoreactivity disappeared from the GABAergic cells with the developmental onset of seizure activity, while the number of the GABAergic neurons remained unchanged [48,49]. When seizures were induced in rats by kainate injection, loss of Pv immunoreactivity in the hippocampus was accompanied by degeneration of GABAergic cells and delayed neuronal death in CA1 and CA3 pyramidal cell layers [9,10]. However, kainate is known to induce not only seizure, but also excitotoxic cell death in the hippocampus [10].

The role of Pv in neuroprotection is not clear: some experimental data support the protective role, others do not [16]. The significant downregulation of Pv in MM neurons in our experiments is not supporting an exclusive neuroprotective role, because no neuronal loss, or degeneration were observed. More plausible explanation is that the downregulation of Pv alters the electrical properties of the MM neurons. According to literature data, Pv as a Ca2+ buffer modulates short-term synaptic plasticity, by enhancing depression and decreasing facilitation [11]. The role of Pn in neuroprotection is not clear: some experimental data support the protective role, others do not [16]. The significant downregulation of Pv in MM neurons in our experiments is not supporting an exclusive neuroprotective role, because no neuronal loss, or degeneration were observed. More plausible explanation is that the downregulation of Pv alters the electrical properties of the MM neurons. According to literature data, Pv as a Ca2+ buffer modulates short-term synaptic plasticity, by enhancing depression and decreasing facilitation [11].

The MM contains projection neurons—the GABAergic innervation of the rat MM originates from midbrain sources and from the surrounding hypothalamic areas [3,4,23]. The high Pv content of the MM neurons can be attributed to the numerous Ca2+ conductances, which are operative in the bursting activity of these cells [5]. Possibly, the Pv content buffers the intracellular Ca2+, regulates facilitation and depression [11] and protects against the damaging aftermath of Ca2+ related intracellular cascades. The downregulation of Pv genes and mRNA translation may indicate the deleterious effects of repeated seizures: the MM neurons may become more susceptible to Ca2+ effects, which later, may lead to the damage and death of these cells. The 20.1% decrease of Pv in MM is substantial, taking into account that every Pv molecule has two Ca2+ binding sites [24]. It seems that 12 days of regular, short seizures are not enough to damage these cells, but we cannot rule out that repeated convulsions longer than these presented in our experiments may lead to the degeneration of the mammillary complex: which is a common autopsy finding in long standing human epilepsies [15].
Acknowledgements

This work was supported by the National Scientific Research Fund, Hungary (OTKA T 26584, T 32566, OMFB-01921/2002 to A.M. and OTKA T 34621 to K.G.). The authors would like to thank Mrs. Zsuzsa Ambrus and Mrs. Katalin Lakatos for their skillful technical assistance.

References


A.L. Scotti, G. Kalt, O. Bollag, C. Nitsch, Parvalbumin disappears from GABAergic CA1 neurons of the gerbil hippocampus with seizure onset while its presence persists in the perforant path, Brain Res. 760 (1997) 109–117.


S. Vizi, K. Gulya, Calculation of maximal hybridization capacity (Hmax) for quantitative in situ hybridization: a case study for multiple calmodulin mRNAs, J. Histochem. Cytochem. 48 (2000) 893–904.