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Repeated 4-aminopyridine induced seizures diminish the efficacy of glutamatergic transmission in the neocortex

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ABSTRACT

Systemic administration of the potassium channel blocker 4-aminopyridine (4-AP) elicits acute convulsions. Synchronized tonic–clonic activity develops during the first hour after the treatment. However, subsequent chronic spontaneous seizures do not appear which suggests changes in neuronal excitability. The aim of our present work was to evaluate alterations in the glutamatergic transmission in the somatosensory cortex of rats following daily, brief convulsions elicited by 4-AP treatment. Changes in general neuronal excitability and pharmacological sensitivity of glutamate receptors were tested in *ex vivo* electrophysiological experiments on brain slices. In parallel studies quantitative changes in subunit composition of glutamate receptors were determined with immunohistoblot technique, together with the analysis of kainate induced Co^{2+} uptake. The results of our coordinated electrophysiological, receptor-pharmacological and histoblot studies demonstrated that repeated, daily, short convulsions resulted in a significant decrease of the general excitability of the somatosensory cortex together with changes in ionotropic glutamate receptor subunits. The relative inhibitory effect of the AMPA receptor antagonist, however, did not change. The NMDA receptor antagonist exerted somewhat stronger effect in the slices from convulsing animals. 4-AP pretreatment resulted in the attenuation of kainate induced Co^{2+} uptake, which suggests either reduction in non-NMDA receptors numbers or reduction in their Ca^{2+} permeability. Repeated seizures decreased GluR1–4 AMPA receptor subunit levels in all cortical layers with a relaitve increase in GluR1 subunits. While the principle NR1 NMDA receptor subunit showed no significant change, the staining density of NR2A subunit increased. These changes in ionotropic glutamate receptors are consistent with reduced excitability at glutamatergic synapses following repeated 4-AP induced seizures.

Introduction

Chemical convulsants like kainate (KA), pilocarpine (PILO), pentylenetetrazol (PTZ), bicuculline (BIC) or N-methyl-D-aspartate (NMDA) are regularly used to evoke highly synchronized electrical brain discharges which are accompanied by characteristic epileptiform behavioural symptoms (Turski et al., 1984; Singh et al., 1991; Cavalheiro, 1995; Nehlig and Pereira 1996; Steriade et al., 1998). Treatment of living animals with these convulsants may elicit spontaneous epileptic activity. A single dose of KA or PILO application results in typical tonic–clonic seizure activity and status epilepticus in the acute period, which is followed by a 3–10 week latency phase, and a subsequent epileptic period, with typical spontaneous tonic–clonic seizure discharges. These chronically epileptic animals are characterized by significant neuronal loss, astrocyte proliferation, sprouting of axon collaterals and synaptic reorganization (Mello et al., 1993; Cavalheiro, 1995; Dubé et al., 2000; Medvedev et al., 2000). The effects of BIC or PTZ are less damaging, the treated animals develop generalized spike and wave activity which is maintained for a long period (Neckelmann et al., 1998., Kliouéva et al., 2001). Repeated subthreshold application of convulsants, *e.g.* glutamate, may also result in enhanced synaptic sensitivity according to the kindling model (McNamara et al., 1993). These epileptic discharges are successfully blocked by different glutamate receptor (GluR) antagonists, however, the susceptibility for specific NMDA or AMPA receptor antagonists are different (Berg et al., 1993; Dóczi et al., 1999; Szabados et al. 2001; Gulyás-Kovács et al. 2002).

Systemic administration of a single dose of the potassium channel blocker 4-aminopyridine (4-AP) also elicits acute convulsions (Mihály et al., 1990). On the EEG synchronized tonic–clonic seizures develop during the first hour after the treatment. 4-AP applied intraperitoneally in 5 mg/kg dose provokes hyperexcitability, tremor, head nodding, forepaw tremor, sniffing, salivation, chewing and myoclonus (Fragozo-Veloz and Tapia, 1992; Mihály et al., 2005). Generally one, sometimes two seizure episodes appear, and animals generally recover in 2–4 h following the application of the drug (Fragoso-

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Veloz et al., 1990; Mihály et al., 1990, 2005). Brain slice experiments showed that 4-AP stimulates cortical layers differently, and characteristic activity pattern develops following treatment (Versteeg et al., 1995; Borbély et al., 2006). Ionotropic GluR antagonists effectively block the spontaneous seizures in the middle and deep layers but not in the superficial layer (Yang and Benardo, 2002). Although subsequent chronic spontaneous seizures do not appear as a consequence of repeated application of 4-AP, effects on the excitability and sensitivity of the brain could be supposed. Our parallel hippocampal studies indicated rearrangement of GluR subunits following repeated 4-AP injections (Borbély et al., 2009).

The aim of the present series of experiments was to investigate the influence of repetitive, short convulsions on the somatosensory cortex in rats. Changes in general neuronal excitability and pharmacological sensitivity of AMPA and NMDA receptors to antagonists were tested in *ex vivo* electrophysiological experiments on brain slices. In parallel studies quantitative changes in AMPA and NMDA receptor subunit proteins were determined with immunohistoblot technique, together with the analysis of kainate induced Co^{2+} uptake through activated non-NMDA receptors.

Materials and methods

Experiments were performed on horizontal plain slices of somatosensory cortex prepared from adult, male Wistar rats (150–280 g, Charles Rivers, Budapest, Hungary). The experimental design had been approved by the Animal Care Committees of universities and by the Budapest- and Szeged Animal Health Care Authorities. Rats were kept under constant 12 h light/dark cycle and controlled temperature (22 ± 2 °C). Standard pellet food and tap water were available *ad libitum*. All chemicals were purchased from Sigma if not stated otherwise. GYKI 52466 was the kind gift of I. Tarnawa.

Treatment of animals

4-AP was dissolved in physiological saline and injected every day intraperitoneally for 12 consecutive days. The starting dose was 4.5 mg/bwkg. If rats failed to produce stage 5 seizure according to the Racine-type scale (Racine, 1972), the dose was increased by 5%, and it was further increased by 5% steps if stage 5 seizure failed to develop on the previous day. The maximal dose was 6.65 mg/bwkg. Control rats received daily physiological intraperitoneally for 12 days.

Slice preparation and electrophysiological recording

Electrophysiological recordings were carried out on 64 slices from 18 rats. Animals were decapitated in deep chloral-hydrate anesthesia (350 mg/bwkg), the brain was quickly removed and horizontal slices (400 μm thick) containing the somatosensory cortex and the hippocampus were cut by a vibratome (Fig. 1A). After 1 h regeneration in the incubation solution containing HEPES-buffer (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (6.7 mM) and its sodium salt (2.6 mM), pH: 7.1–7.2) slices were transferred to a Haas-type recording chamber (Experimetria, Budapest, Hungary) through which standard artificial cerebrospinal solution (ACSF) was perfused (1.5 ml/min) using a peristaltic pump (Heidolph PD 5101). The solution was saturated with carbogene (5% CO_2–95% O_2) at 33 ± 1 °C. The composition of this standard perfusion ACSF solution was (in mM): 126 NaCl; 26 $NaHCO_3$; 1.8 KCl; 1.25 KH_2PO_4; 1.3 $MgSO_4$; 2.4 $CaCl_2$; 10 glucose.

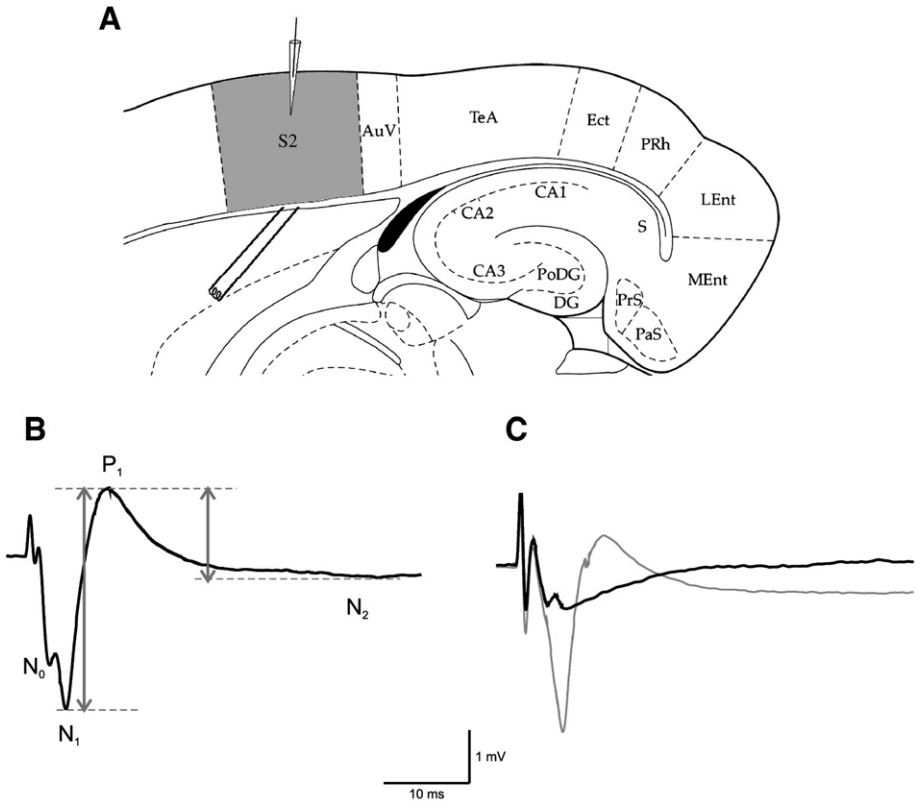

Fig. 1. (A) The schematic diagram indicates the site of recording (lower part of layer 3 of the somatosensory cortex) and the position of the bipolar stimulation electrode right below the recording electrode at the border of the white and grey matters (modified from Paxinos and Watson, 1982). S2: the somatosensory area. (B) Original record of field response evoked by electrical simulation. The first part (N0) of the early negative wave corresponded to the incoming action potentials, which was followed by a synaptic (N1) peak. N0 is frequently not possible to distinguish as a separate peak; usually it only appears as a small deflection on the leading edge of the potential. P1 the first positive and N2 the second negative peaks. The evoked responses were characterized by the peak-to-peak amplitude of the early (N1–P1) and late (P1–N2) components. (C) Original record of evoked field response (grey line) which demonstrates that both (N1–P1) and (P1–N2) peaks are effectively lessened by the mixture of 40 μM GYKI 52466 and 25 μM APV after 30 min incubation (black line).

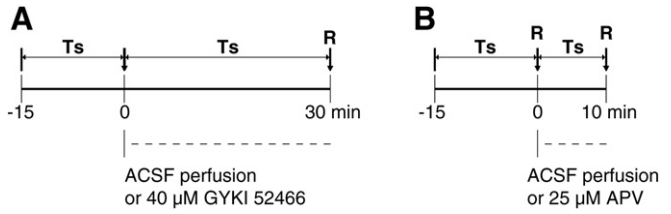

Fig. 2. Schedule of different treatments during the recordings from slices. –15 means the beginning of the incubation of slices in the recording chamber when continuous test stimulation was applied. Basic activity was recorded at 0 min, and than at half of the slices the perfusion solution was switched to glutamate antagonist containing solution. R: recording points, when I–O curves and average of 10 evoked responses (aER) at 2 T stimulation intensity were recorded. Ts: test stimulation at 2 T stimulus intensity with 0.05 Hz frequency.

Glass microelectrodes filled with 1 M NaCl (8–10 MΩ) were positioned as recording electrodes into the lower part of layer III of the somatosensory cortex, while bipolar tungsten stimulating electrodes were positioned right below the recording electrodes at the border of the white and grey matter. Duration of the stimulation square voltage pulses was 100 μs and the amplitude was gradually varied between threshold and supramaximal values. Signals were amplified with an Axoclamp 2A amplifier (Axon Instruments Inc., Union City, CA), A/D converted and recorded with the SPEL Advanced Intrasys computer program (Experimetria Ltd., Budapest, Hungary).

The viability of each slice was tested at the beginning of the procedure. Single-shock stimulation evoked a characteristic field response, which consists of an early and a late component (Fig. 1B). In accordance with the analysis of Abbes et al. (1991) and Hodgson et al. (2005) the first part (N0) of the early negative wave corresponded to the incoming action potentials, which was followed by a synaptic (N1) peak. Average latencies of N0 and N1 peaks were 3.7 ms and 6.1 ms, respectively. N1 usually was of higher amplitude compared to N0. In our experiments N0 was not always possible to distinguish as a separate peak; usually it only appeared as a small deflection on the leading edge of the potential. The early negative component was followed by a positive (P1) peak and they were effectively inhibited by the mixture of GYKI 52466 and APV (Fig. 1C).

If the peak-to-peak amplitude of the maximal evoked response (N1–P1) was smaller than 1 mV, the slice was not used for further experiments. Slices were continuously stimulated with medium-strength stimuli at a rate of 0.05 Hz. 15 min after placing the slices into the recording chamber the stimulus threshold (T) was determined. Then input–output (I–O) curve was recorded when gradually increasing the stimulus intensity from T to 4 T in 6 steps. Response amplitudes were plotted against intensities, and than an average of 10 evoked responses (aER) at 2 T stimulation intensity was recorded. At each stimulus intensity mean amplitude value were calculated from three evoked responses. Subsequently, 0.05 Hz continuous stimulation was applied with 2 T if not specified otherwise, so called test response (TS) evoked at this stimulation intensity were calculated as a

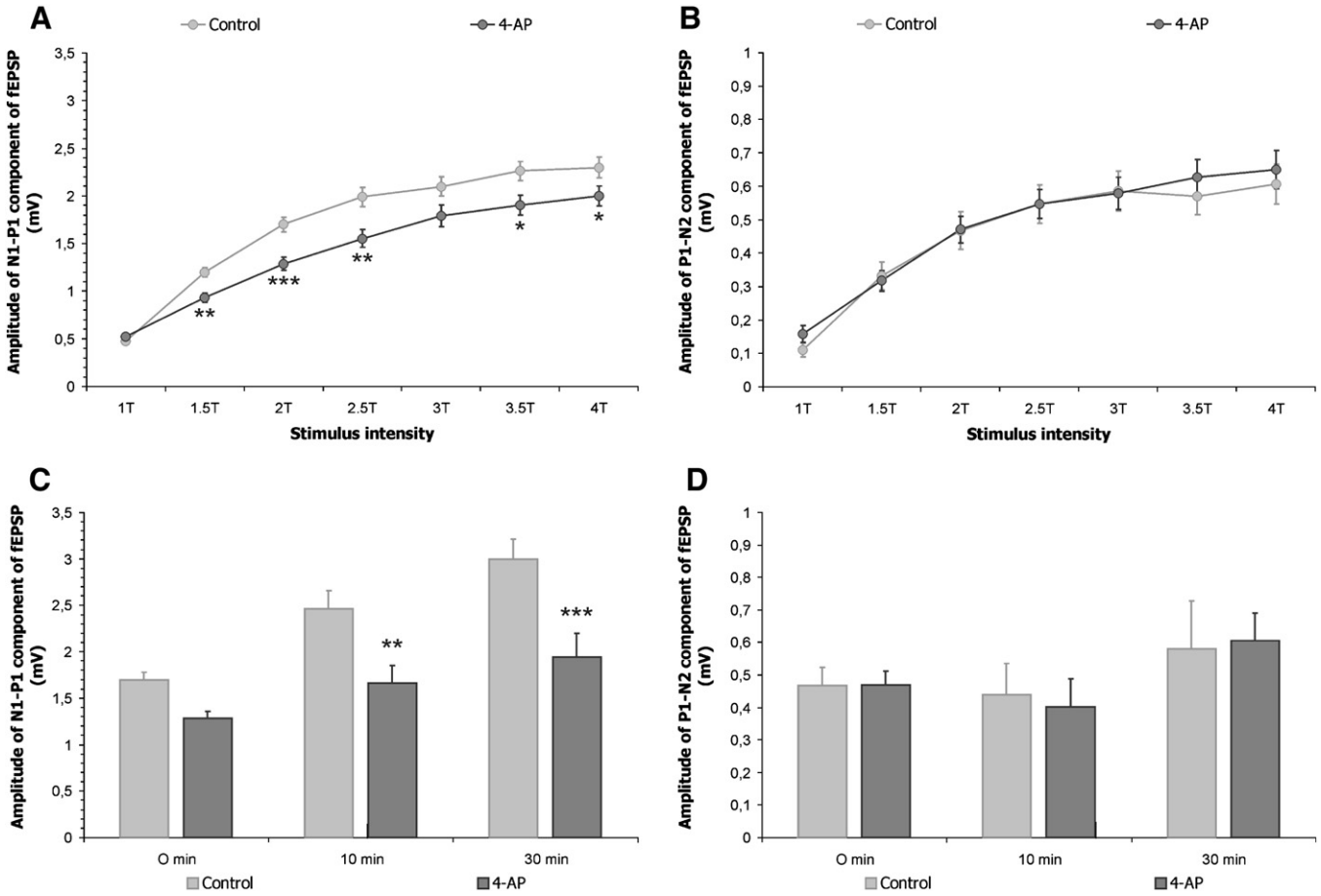

Fig. 3. Alterations of basic excitability in the somatosensory cortex slices after the 12-day-long 4-AP pretreatment. (A) Changes in peak-to-peak amplitude of the early (N1–P1) components of the evoked response and (B) changes of the amplitude of the late (P1–N2) component against the gradually increasing stimulus intensity are represented. Continuous 10 min test stimulation at 2 T stimulus intensity resulted in changes of amplitude of evoked response (aER). (C) After 30 min of a 0.05 Hz, stimulation this increase was enhanced. However, 4-AP pretreatment lessened the amplitude of the evoked response in all experimental groups. (D) The 4-AP pretreatment had no detectable effect on the P1–N2 component. (T means the stimulus threshold of evoked response. * symbol indicates significant differences at $p<0.05$, while ** means $p<0.01$, and *** means $p<0.001$).

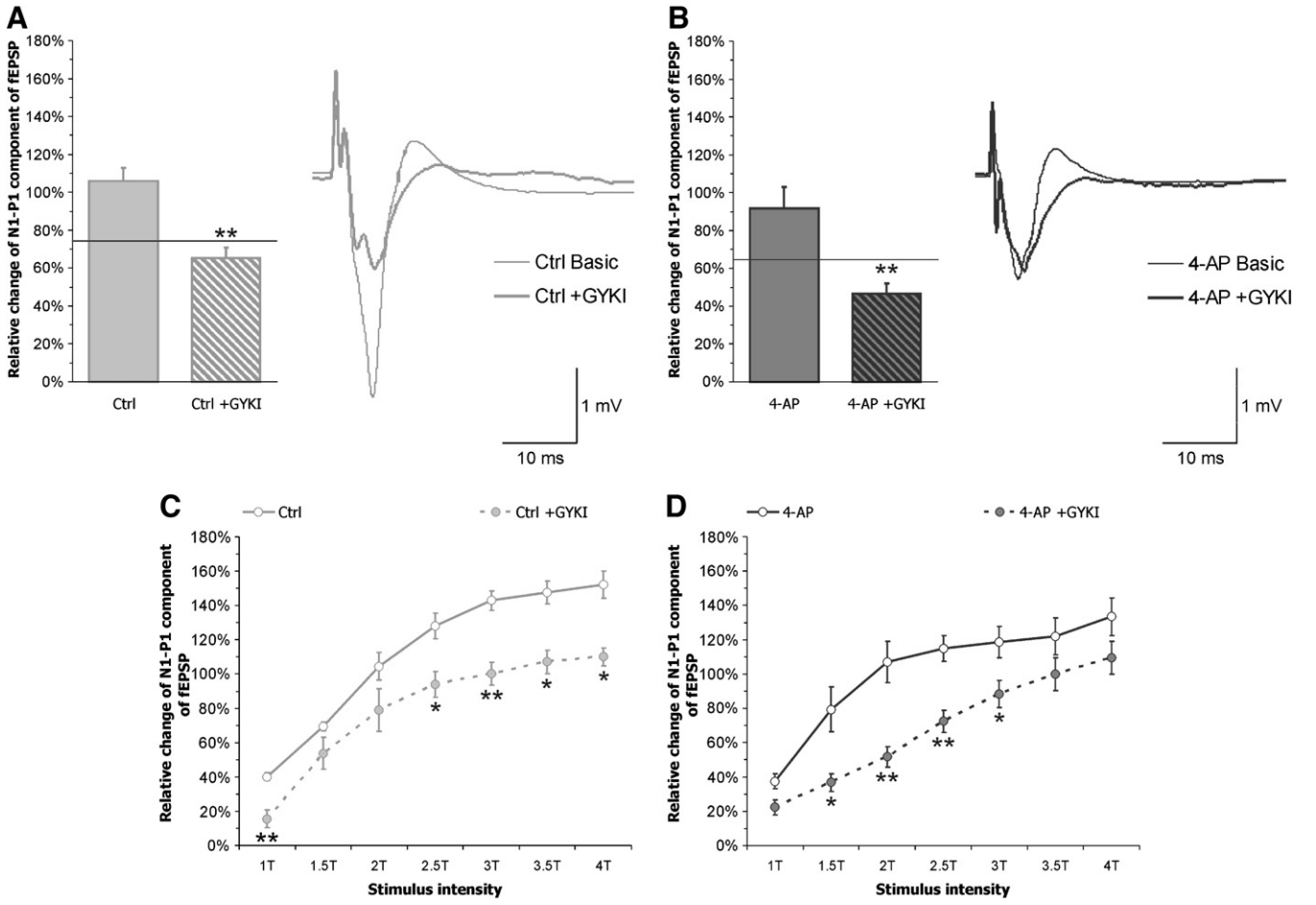

Fig. 4. Effect of AMPA receptor antagonist GYKI 52466 on the amplitude of early (N1–P1) component of the evoked response (aER) in control (A) and 4-AP pretreated (B) slices. Columns demonstrate significant inhibition at 2 T stimulus intensity in both groups, the effects were, however, more enhanced in the control group. Graphs demonstrate original records. C and D show the whole stimulus intensity-evoked response (I–O) curves. T means the stimulus threshold of evoked response. * symbol indicates significant differences at $p<0.05$, while ** means $p<0.01$.

mean of ten evoked responses. Depending on the aim of the tests different protocols were followed (Fig. 2). To test the altered basic excitability following chronic 4-AP treatment no special treatment was applied apart from the continuous low frequency stimulation. To analyze pharmacological sensitivity of particular GluRs, standard perfusion solution was switched either to AMPA receptor antagonist containing solution (40 μM GYKI 52466; 1-(aminophenyl)-4-methyl-7,8 methylendioxy-5H-2,3-benzodiazepine), or to NMDA receptor antagonist containing solution (25 μM D-APV; D-2-amino-5-phosphonopentanoic acid). Recordings were finished by determination of test responses and I–O curves (Figs. 2A, B). Each experimental group contained 8–8 independent records.

Recorded data were analyzed also with the SPEL Advanced Intrasys computer program (Experimetria Ltd., Budapest, Hungary). The short latency, early component was characterized by the peak-to-peak amplitude of N1–P1 waves. The longer latency, late component was characterized by the peak-to-peak amplitude of the P1 and the second negative (N2) component (Fig. 1B).

Statistical analysis

To compare control and treated groups, in each series of experiments one-way ANOVA was performed followed by Newman–Keuls post hoc test ($p<0.05$). Homogeneity of variances and normal distribution of data were tested before statistical analysis. Data were presented as mean ± S.E.M.

Cobalt-uptake determination

Slices were obtained form nine control and ten 4-AP treated animals to test alterations in Co^{2+} uptake. Slice preparation was carried out in the same way as described for electrophysiological recordings. The thickness of the slices was, however, 250 μm. From each rat 8–10 horizontal slices containing the somatosensory cortex slices were stained and evaluated. The procedure was performed as described previously (Pruss et al., 1991). Briefly, slices were incubated in a Ca^{2+} free incubation solution for 5 min before placement into an uptake buffer (13 mM sucrose, 57.5 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 12 mM glucose, 10 mM HEPES) supplemented with 5 mM $CoCl_2$ and 100 μM KA. Incubation lasted for 20 min at room temperature (~20 °C). Slices incubated in the same solution without KA served as background controls. After the stimulated Co^{2+} uptake, slices were rinsed once in the uptake buffer and incubated in the same buffer containing 2 mM EDTA to remove non-specifically bound Co^{2+}. Following that, slices were rinsed twice with the uptake buffer, and Co^{2+} was precipitated by incubation in a 0.12% $(NH_4)_2S$ solution for 5 min. During this procedure, dark CoS precipitate was formed in the cells. At the end, slices were fixed in 4% paraformaldehyde for 30 min and mounted into glycerin for image analysis. Co^{2+} stained slices were analyzed by an Olympus CH-2 microscope equipped with an Olympus Camedia C4040-Zoom digital camera connected to a PC running an AnalySIS 3.2 Docu (Soft Imaging System) software. Pictures were digitized at a 4-fold magnification and converted to 8-bit grey scale

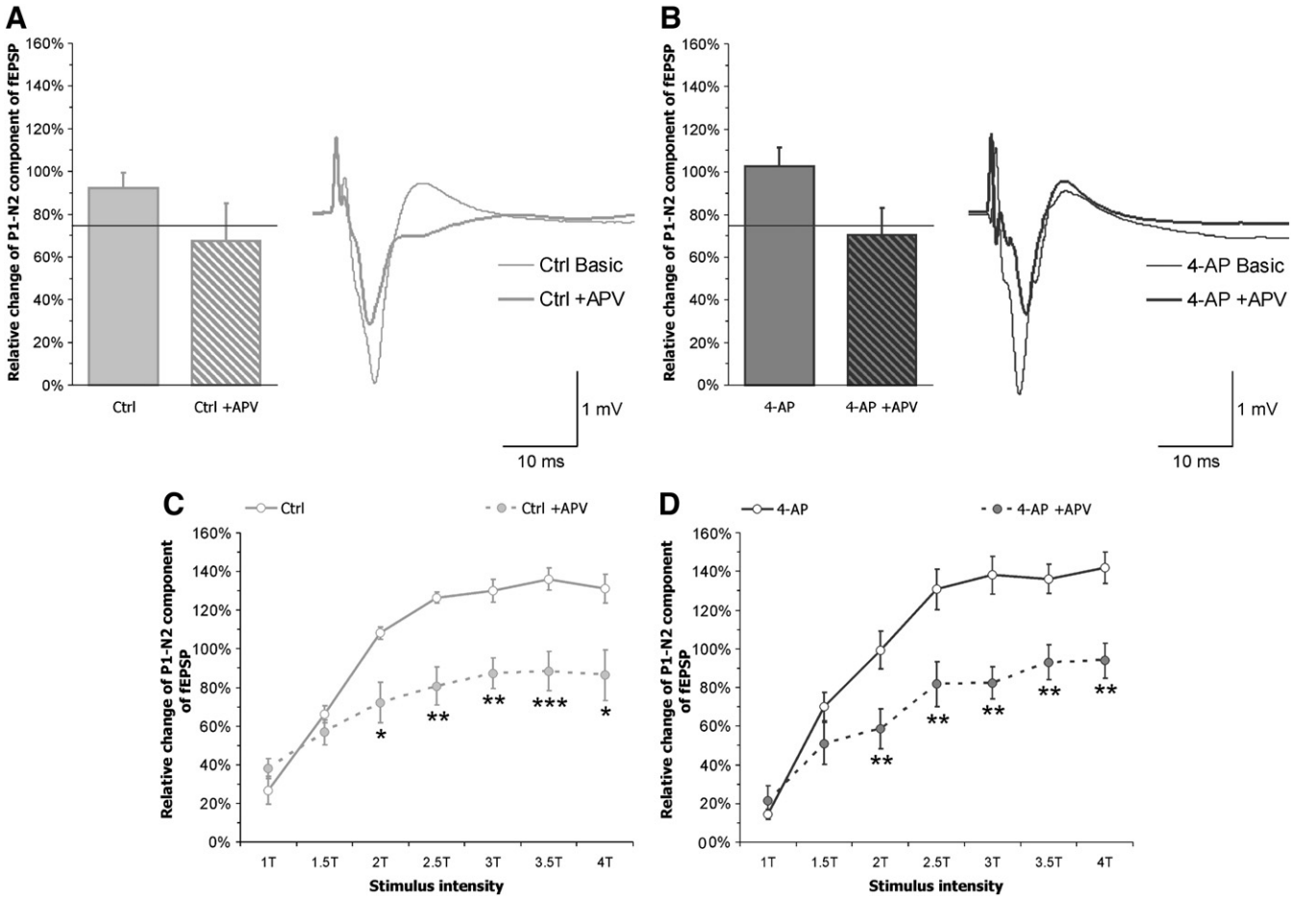

Fig. 5. Effect of NMDA receptor antagonist APV on the amplitude of the late (P1–N2) component of the evoked response (aER) in control (A) and in 4-AP pretreated (B) slices. Columns demonstrate the effects at 2 T stimulus intensity in both groups, the effects was, however, slightly enhanced in the control group. Graphs demonstrate original records. C and D show the whole stimulus intensity-evoked response (I–O) curves. T means the stimulus threshold of evoked response. * symbol indicates significant differences at $p<0.05$, while ** means $p<0.01$, and *** means $p<0.001$.

images as follows: perpendicular to the pial surface a histogram was taken between the I. cortical layer and the corpus callosum. According to the thickness of the cortical region each recorded area was 50 pixel thick and 300–600 long. Staining intensity was measured in arbitrary units; one data point corresponded to the mean of 50 recorded points. Optical density (OD) of a 50×100 pixel area in the corpus callosum was determined in each slide as background and data were always corrected with this OD value. In all series of experiments the corrected OD values of untreated slices were subtracted from the corrected OD values of KA stimulated slices. Parallel control and 4-AP treated slices were compared. For this comparison the distance between the pial surface and the corpus callosum was divided to 20 equal divisions, which were correlated to cortical layers on the basis data of Skoglund et al. (1996). The borders of each layer (L) were as follows: LI: 1. division, LII: 2–3 division, LIII: 4–6 division, LIV: 7–8 division, LV: 9–13 division, LVI: 14–20 division.

Histoblot analysis of ionotropic glutamate receptor proteins

Rats were sacrificed on the day following the final 4-AP treatment, and used to determine changes in the distribution of different AMPA and NMDA type GluR subunit proteins, using an *in situ* blotting technique (Tonnes et al. 1999; Gallyas et al. 2003; Kopniczky et al., 2005). In brief, animals were deeply anesthetized with diethyl ether, decapitated and the brains were quickly frozen in isopentane and stored at −80 °C until sectioning. Horizontal cryostat sections (10 μm) were apposed to nitrocellulose membranes, which were previously moistened with 48 mM Tris-base, 39 mM glycine, 2% (w/v) SDS and 20% (w/v) methanol for 15 min at room temperature (~20 °C). After blocking in 5% (w/v) non-fat dry milk in PBS, nitrocellulose membranes were DNase I-treated (5 U/ml), washed and incubated in 2% (w/v) SDS, 100 mM β-mercaptoethanol in 100 mM Tris–HCl (pH 7.0), for 60 min at 45 °C to remove adhering tissue residues. After excessive washing, blots were reacted with affinity-purified subunit specific antibodies (0.5 μg/ml) in blocking solution overnight, at 4 °C. The following primary antibodies were used: anti-GluR1; anti-GluR2 (Chemicon; 1 μg/ml); anti-GluR1-4 (pan-AMPA, Pickard et al. 2000), anti-NR1 (Pharmingen; 2 μg/ml); anti-NR2A (Chemicon; 1 μg/ml); anti-KA-2 (Upstate; 1:500). The bound primary antibodies were detected with alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (Tonnes et al. 1999). To facilitate the identification of structures and cell layers, adjacent cryostat sections were stained with cresyl violet. Digital images were acquired by scanning the membranes using a desktop scanner, data were stored as grey scale images. Evaluation of images was carried out according to method described at cobalt-uptake determination.

Results

Seizure behaviour

The i.p. administration of 4-AP induced generalised tonic–clonic seizures. In average, one or two seizures were detected following the convulsant application. The average latency of the first seizure was

26.6 ± 5.4 min. This event was frequently followed by a second fit within the first hour after the convulsant treatment. The mean delay between the two seizure activities was 16.2 ± 4.9 min.

General neuronal excitability

Results show a general decrease in excitability following chronic 4-AP pretreatment. The basic I–O curves (Fig. 3A) demonstrate that the mean amplitude of the early component is reduced compared to control slices. At 2 T stimulus intensity the aER was 1.8 ± 0.08 mV in the control v.s. 1.4 ± 0.08 mV in the 4-AP pretreated slices. Following test stimulation without any treatment the amplitude increased in both experimental groups, but it remained significantly smaller in the 4-AP pretreated slices. After 10 min the amplitudes were 2.46 ± 0.19 mV (136.6%) vs. 1.66 ± 0.19 mV (115.3%), while after 30 min they were 2.97 ± 0.19 mV (165.0%) vs. 1.94 ± 0.26 mV (138.6%) (Fig. 3C). The late component did not always appear in the control slices or its amplitude was very small (0.46 ± 0.06 mV), which was the same (0.47 ± 0.04 mV) in the 4-AP pretreated slices (Fig. 3B). After 10 min incubation it was 0.45 ± 0.95 mV and 0.41 ± 0.09 mV, and after 30 min incubation it reached 0.58 ± 0.14 mV and 0.60 ± 0.09 mV, respectively (Fig. 3D).

Pharmacological sensitivity to AMPA and NMDA receptor antagonists

Application of GluR antagonists into the perfusion solution effectively reduced the amplitude of the evoked responses. The mixture of GYKI 52466 and APV lessened the amplitude of all components, the most sensitive part was the P1 wave. The diminish of N1–P1 peak was 81 ± 6.1% following the application of 40 μM GYKI 52466 together with 25 μM APV for 30 min (Fig. 1C). Application of the AMPA receptor antagonist GYKI 52466 alone for 30 min into the

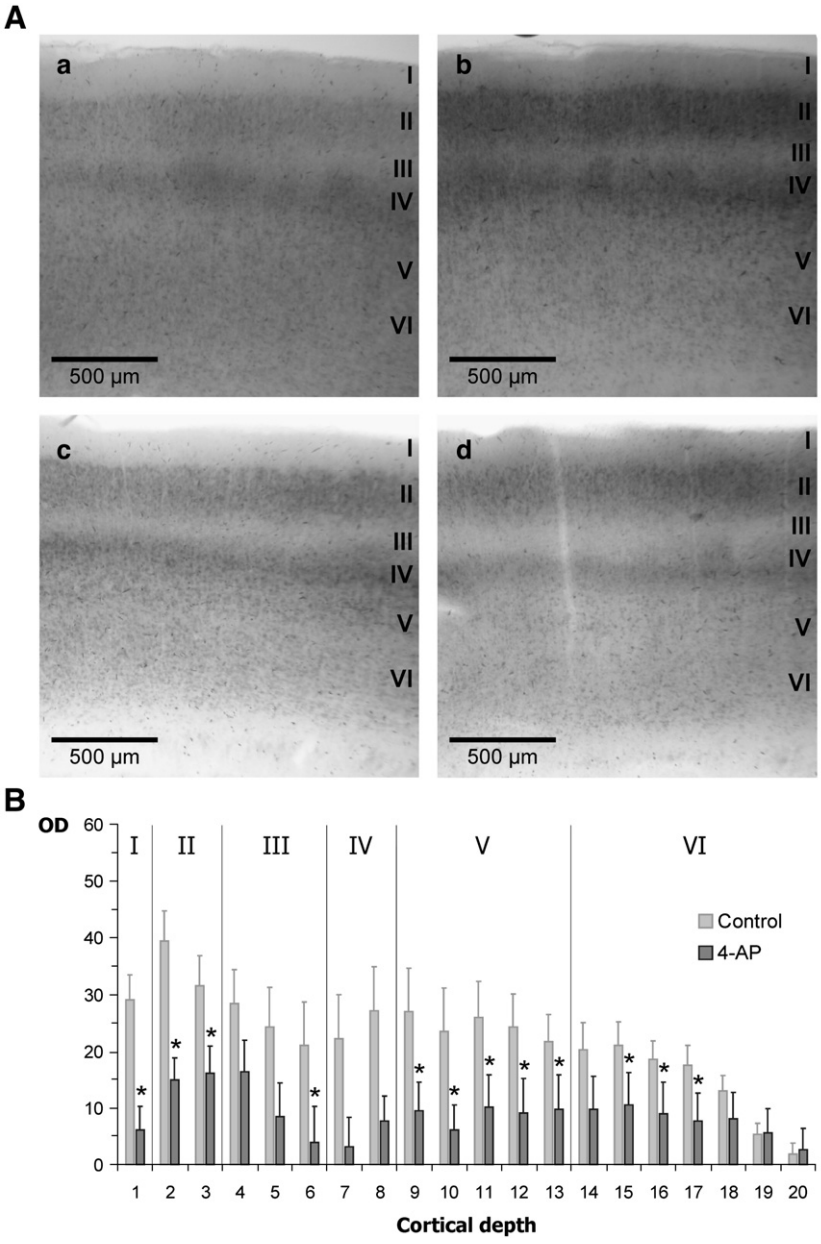

Fig. 6. The effect of the 4-AP pretreatment on KA induced Co^{2+} uptake in different layers of the sensory cortex. (A) Figures show the original records from control (a, b) and from the 4-AP pretreated (c, d) slices, a and c panels represent the background activity, while b and d panels represent samples following KA treatment. The difference of the optical density (OD) between background and KA treated slices were calculated along the cortex, perpendicular to the pial surface and the corpus callosum. These differences in OD are demonstrated on (B) part of the figure. I–VI represent the proper cortical layers. Overall Co^{2+} uptake of the 4-AP pretreated slices is reduced compare to control slices. * symbol indicates significant differences at $p<0.05$.

perfusion solution reduced the increment of peak-to-peak amplitude both in the control and in the 4-AP pretreated slices (Fig. 4). The relative effectiveness of the inhibitor on the N1–P1 peak was smaller in the 4-AP pretreated group. In the control slices, the amplitude of the evoked response at 2 T stimulus intensity was 105.9 ± 7.1% of the basic maximal amplitude. In the presence of GYKI 52466 the relative value was only 65.4 ± 5.5%. In 4-AP pretreated slices the amplitude at 2 T stimulus intensity was 91.5 ± 11.4% of the basic maximal amplitude, and in the presence of GYKI 52466 it reached 46.7 ± 5.4%.

The NMDA receptor antagonist APV reduced the amplitude of the early and the late component of the evoked responses (Fig. 5), and typically decreased the P1–N2 wave. While the relative amplitude value was 92.2 ± 7.3% in the control and 102.6 ± 8.8% in the 4-AP pretreated slices after 10 min incubation in normal ACSF, in the presence of APV the amplitude values reached 67.3 ± 17.8% in the control and 70.2 ± 12.8% in the 4-AP pretreated slices.

Modification in Co^{2+} uptake through AMPA/KA receptors

KA effectively activates non-NMDA receptors and Co^{2+} can cross the plasma membrane through Ca^{2+} permeable ionotropic GluR channels. The difference in the optical densities (OD) between background and KA treated slices was used to estimate the abundance of Ca^{2+} permeable AMPA/KA receptor population in sensory cortical regions. Overall, OD associated with Co^{2+} uptake along the cortex calculated perpendicular to the pial surface and the corpus callosum was higher in control slices than in slices obtained from 4-AP treated rats (Fig. 6), the mean alteration was 39 ± 14%. These differences appeared in each layer of the somatosensory cortex, which indicated a significant reduction in the density of functional, Ca^{2+} permeable AMPA/KA receptors in 4-AP pretreated slices.

Alteration in glutamate receptor subunit densities

To complement the functional studies, histoblot analysis was performed to investigate changes in ionotropic GluR subunit protein levels in different cortical layers. Pooled data is illustrated on Fig. 7 and in the text below we indicate % changes in cortical layer III, where the recording electrodes were positioned in the electrophysiological experiments. Changes in total AMPA receptor expression levels were investigated with a polyclonal antibody recognizing the conserved extracellular loop region of all four AMPA receptor subunits (GluR1–4, both *flip* and *flop*; Pickard et al., 2000). GluR1–4 levels decreased in all cortical layers (89 ± 2% in layer III), which is consistent with reduced glutamatergic transmission established in our functional studies (Fig. 7). To identify differential changes in individual AMPA receptor subunit proteins, we performed additional experiments with GluR1 and GluR2 selective immunoreagents. The relative density of GluR1 AMPA receptor subunit protein increased significantly in layers III (113 ± 9%). The density of GluR2 subunit was not altered in layers I–V of the somatosensory cortex. However, a small but significant reduction in GluR2 immunoreactivity was detected in deeper segments of layer VI (Fig. 7). Due to the lack of sufficiently specific GluR3 antibody, we were unable to establish changes in the expression level of this subunit. The expression level of GluR4 is low in the cerebral cortex throughout development (Molnar, 2008) therefore it is unlikely that

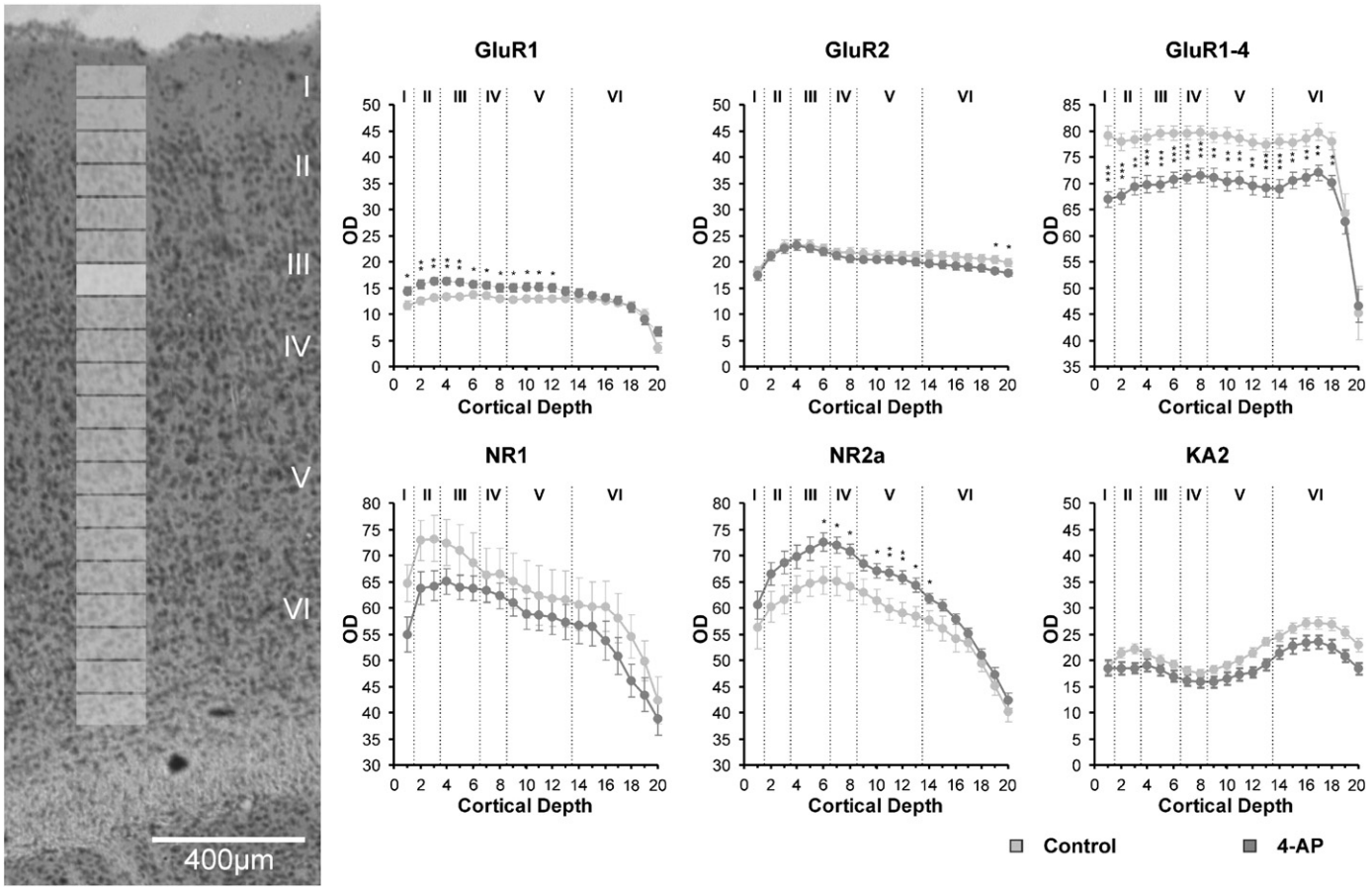

Fig. 7. 4-AP treatment induced differential changes in ionotropic GluR subunit densities. The picture represents part of the somatosensory cortex following Nissl staining. Areas highlighted in grey correspond to the regions where changes in optical density were measured in immunostained histoblots. The light grey cube symbolizes the place where the recording electrodes were positioned in the electrophysiological experiments. Graphs show the densities of different ionotropic GluR subunit proteins after the 12-day-long 4-AP pretreatment. *$p<0.05$ level, **$p<0.01$, ***$p<0.005$. The number of independently tested brain sections (the "*n*" values) are as follows: NR1: 24; NR2a: 24; NR2b: 20; GluR1: 26; GluR1–4: 24; $GluR1_{flop}$: 24; GluR2: 20; KA-2: 36.

this subunit plays a significant role. Concerning the NMDA receptor subunits, there was a moderate reduction in NR1 subunit densities (91 ± 4% in layer III), but it was not significant. On the other hand, there was an increase in NR2A expression levels in layers III (109 ± 3%). There were no detectable changes in KA-2 KA receptor densities in brain samples obtained from 4-AP treated rats (96 ± 3% in layer III). In summary, the histoblot analysis indicates significant changes in ionotropic GluR proteins which include a prominent down-regulation of total AMPA receptors in all cortical regions and differential changes in AMPA and NMDA receptor subunits which may alter their subunit composition in different cortical layers.

Discussion

The aim of our present work was to evaluate alterations in the glutamatergic transmission following daily, brief convulsions elicited by 4-AP treatment. The results of our coordinated electrophysiological, receptor-pharmacological and histoblot studies demonstrated that repeated convulsions for 12 days result in a significant decrease of the excitability of the somatosensory cortex together with the rearrangement of AMPA and NMDA receptor subunits. The general excitability of the somatosensory cortex decreased, the mean amplitude of the field potentials evoked by electrical stimulation was significantly smaller after the 4-AP pretreatment compared to the control. The relative inhibitory effect of the AMPA receptor antagonist GYKI 52466, however, did not change after the treatment. The NMDA receptor antagonist APV exerted somewhat stronger effect in the slices from convulsing animals. 4-AP pretreatment resulted in the attenuation of KA induced Co^{2+} uptake, which suggests either reduction in the number of non-NMDA receptors or reduction in their Ca^{2+} permeability. Repeated seizures resulted in a decrease in the immunoreactivity of GluR1–4 AMPA receptor subunits in all cortical layers, while the density of the GluR1 subunit increased in outer layers of the cortex. As to the NMDA receptor subunits, the staining density of NR2A increased without significant change in NR1 protein expression.

In the majority of vertebrate central nervous systems ionotropic glutamate receptor channels mediate fast excitatory synaptic responses (Dingledine et al., 1999, Mayer, 2005). Several experimental data show that electrical stimulation or application of different chemical convulsants result in alteration of synaptic efficacy (Barnes and Pinel, 2001; Rejdak et al., 2001; Addae et al., 2007). Depending on the characteristics and intensity of stimulation synaptic adaptations can develop to different directions. On one hand, neuroprotective processes may initiate and brain tolerance develops, or on the other hand following the severe over-excitation neuronal degeneration may emerge. Changes in GluR characteristics are often detected subsequent to seizures or other neuronal injuries (Michaelis, 1998; Világi et al., 1999). Application of repeated mild electrical stimulation elicits generalized tonic–clonic seizures. The underlying molecular and cellular processes are not fully understood, but it was shown, that synaptic efficacy increases and neuronal networks reorganize in this epilepsy model (Morimoto et al., 2004). Large dose of intraperitoneal or cerebroventricular KA exposure also results in development of severe seizure. It is proven that following these serious insults the subunit composition of non-NMDA GluRs change (Grooms et al., 2000; Jia et al., 2006), which may lead to increased Ca^{2+} influx into activated neurons. Ca^{2+}-triggered excitotoxic processes may be initiated, which either result in local rearrangements at synapses or induction of cell death (Stone and Addae, 2002; Liu and Zukin, 2007). Glial and neuronal glutamate transporters play important role in the regulation of extracellular glutamate level. Alteration in transporter function may also be noticed during convulsions (Furuta et al., 2003; Campbell and Hablitz, 2008). In addition to excitotoxicity, neuroprotective processes may also be triggered by increased synaptic activity in seizures (Mattson, 2007).

4-AP is a commonly used convulsant. It selectively blocks some types of voltage gated K^+ channels (Thompson, 1982; Den Hertog et al., 1987; Kiss et al., 2002), which cause neuronal depolarization and facilitate Ca^{2+} influx into presynaptic terminals enhancing the transmitter release (Thesleff, 1980; Qian and Saggau, 1999; Hu et al., 2001; Gu et al., 2004). As the ratio of the excitatory glutamatergic transmission in the neocortex is very high (Rubenstein and Merzenich, 2003), following 4-AP application epileptiform seizure develops as cortical activity is pushed toward excitation (Szente and Baranyi 1987; Fragozo-Veloz et al., 1990). However, acute seizure initiated by 4-AP is not followed by spontaneous recurrent seizures in the chronic phase. Although some data point to receptor reorganization, major structural or biochemical rearrangements were not detected following long lasting 4-AP application (Vizi et al., 2004).

Our electrophysiological results show that seizure activity, evoked by repeated daily application of 4-AP, triggers neuroprotective mechanisms in the somatosensory cortex diminishing basic excitability. High amount of glutamate released into the extracellular space during seizures may initiate these neuronal protective processes, i.e. AMPA receptor desensitization or internalization (Greger and Esteban, 2007), which might result in the decreased excitability. It was also recently reported that neuroprotective processes may be initiated through NMDA receptor activation, application of mild convulsant preconditioning stimuli results in neuronal tolerance and attenuates the effects of further serious insults (Rejdak et al., 2001). It is also proved that following epileptic seizures or other brain injuries the concentration of endogenous kynurenic acid increase. Kynurenic acid appears to be released by glial cells and protects neurons by antagonizing the activation of ionotropic Glu receptors (Wu and Schwartz, 1996; Scharfman et al., 1999). These processes may cause the reduction of peak-to-peak amplitude of the early (N1–P1) component of the electrically evoked field potential in slices from pretreated animals. Normally these parts of the excitatory field potential are the largest in layer III of the somatosensory cortical slices and develop with characteristic latency as a consequence of synaptic activation of neurons in the recorded layer (Abbes et al., 1991). As non-NMDA receptor activation plays a crucial role in the development of the early component of the evoked response (Conti and Weinberg, 1999), reduced amplitude to be seen in I–O curve of pretreated slices refer to retarded AMPA receptor activity. The AMPA receptor antagonist GYKI 52466 (Tarnawa et al., 1990) effectively inhibited the evoked response also in the pretreated slices showing that AMPA receptors were not fully suppressed. In the background of the reduced excitability there might be a decrease in the sensitivity- or a reduction in number of active AMPA receptors. The overall Co^{2+}-uptake activated by KA was also depressed in all layers, while the characteristic pattern of the laminar receptor distribution (Zilles et al., 1999) did not change. So we can conclude that the number of functional non-NMDA receptors in the postsynaptic membrane decreased. Strong activation during the epileptic seizures may drive internalization of AMPA receptors, and repetitive stimulation may initiate metabolic processes to depress the synaptic efficacy (Lüscher and Frerking, 2001). Results of our histoblot studies also support this notion. Decrease in the amount of GluR1–4 subunits in all cortical layers is consistent with the reduced basic excitability and the lessening of the amplitude of early component of filed potential. Differential changes in relative expression levels of GluR1, GluR2 AMPA receptor and NR1, NR2A NMDA receptor subunits in some of the cortical layers suggest 4-AP treatment induced subunit rearrangements in ionotropic GluRs. It is possible that the relative increase in NR2A subunit containing NMDA receptors also contribute to the depression of glutamatergic synaptic transmission (Schotanus and Chergui, 2008).

The same treatment may evoke dissimilar responses in different brain regions which can lead to either sensitization or depression. These plastic changes may depend on the characteristic function of

the investigated area. On the basis of our experiments, we can conclude that in the somatosensory cortex over-excitation may initiate tolerance, which can guarantee the protection of this functionally highly significant brain region from extensive damage.

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