Differential gene expression in acute and chronic seizure models in the rat brain

Ph.D. Thesis

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ABBREVIATIONS

AP-1	activator protein -1
AD	afterdischarge
ANOVA	analysis of variance
AOI	areas of interest
4-AP	4-aminopyridine
DAB	3,3-diaminobenzidine tetrahydrochloride
ECL	enhanced chemiluminescence method
ECS	electroconvulsive shock
EPSC	excitatory postsynaptic current
GABA	gamma aminobutyric acid
IEG	immediate early gene
IHC	immunohistochemistry
IPSP	inhibitory postsynaptic potential
LECA	lateral entorhinal cortex ablation
MM	medial mammillary nucleus
NMDA	N-methyl-D-aspartate
nRt	thalamic reticular nucleus
PAP	peroxidase-antiperoxidase
PBS	phosphate buffered saline
PMSF	phenylmethanesulfonyl fluoride
PV	parvalbumin
rCBF	regional cerebral blood flow
RT	room temperature
RT-PCR	reverse transcriptase–polymerase chain reaction
SE	status epilepticus
SEM	standard error of means
SOC	sham-operated control
TF	transcription factors
TLE	temporal lobe epilepsy

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INTRODUCTION

1. Seizures and epilepsy

Epilepsy encompasses a number of different syndromes characterized by the recurrent appearance of spontaneous seizures. The term ’seizure’ refers to a transient change of behaviour due to the disordered, synchronous, and rhythmic firing of populations of neurons in the central nervous system (McNamara, 1994).

Epileptic seizures are generally classified into partial and generalized syndromes. In generalized epilepsies, the predominant type of seizure begins simultaneously in both cerebral hemispheres. Absence, myoclonic and tonic-clonic are examples of generalized seizures. Many forms of generalized epilepsy have a strong genetic component, some involve mutations in genes encoding ion-channel proteins (i.e. sodium-channel subunits, potassium-channel subunits). Since these genes are expressed throughout the brain, it is plausible that the effect of the mutations is diffuse and therefore confers a predisposition to a generalized seizure disorder (Chang et al., 2003).

Partial seizures originate in one or more localized foci, usually as a result of central nervous system insults such as after head trauma, strokes, tumors. The clinical manifestation of focal seizures varies depending upon the origin of epileptic discharges, and includes motor, sensory, autonomic, and mental symptoms. Seizures are defined as simple partial if there is no loss of consciousness, and complex partial if there is a loss of consciousness. When the seizure discharge becomes sufficiently widespread, and includes a strong participation of motor system circuitry, the result is a convulsive response that typically includes both tonic (sustained contractions) and clonic (oscillating contractions and relaxations) components (Morimoto et al., 2004).

Temporal lobe epilepsy (TLE) is the most common and drug-resistant type of adult focal epilepsy. The epileptic focus in TLE patients often resides in mesial temporal structures, such as the hippocampus or amygdala, and the seizures are complex partial, typically accompanied by motionless staring and oroalimentary automatisms (Engel, 1996). Although the etiologies of TLE are heterogeneous, patients with mesial TLE tend to have a past history of prolonged febrile seizures or status epilepticus. The surgically resected hippocampus from drug-resistant TLE patients often shows degenerative effects, called hippocampal sclerosis, including gliosis and a loss of neurons. In addition to these degenerative changes, the epileptogenic

hippocampus demonstrates neuronal reorganization, as evidenced by measures of axonal sprouting and synaptogenesis (Sutula et al., 1989).

2. Experimental models of seizure and epilepsy

In the investigation of the basic mechanisms that govern epilepsy, work on experimental animals provides a more available tool than work on human material.

Acute experimental models use chemical or electrical stimulations applied to normal brain, being useful models of convulsions rather than of epilepsy.

Chronic animal models must change the structure and/ or function of the brain to make it genuinely epileptic, that is, performing spontaneous recurrent seizures. Examples of chronic epilepsy models include kindling or several models that trigger status epilepticus, i.e. by administration of kainic acid or pilocarpine and a subsequent relapse into recurrent seizures after a latent period (Morimoto et al., 2004).

2.1. Experimental models of epileptogenesis

Kindling is still widely accepted as a functional epilepsy model in which the altered neuronal response develops in the absence of gross morphological damage, such as that seen in many other epilepsy models. Kindling presents itself as an apparently simple phenomenon in which repeated induction of focal seizure discharge produces a progressive, highly reliable, permanent increase in epileptic response to the inducing agent, usually electrical stimulation (Racine, 1978).

In limbic kindling, the initial focal seizure response is expressed mainly in the epileptiform discharge triggered at the stimulated site. The only behavioral response during the early stages of kindling is a freezing response during the evoked ictal discharge. With repeated activation, the seizure response becomes generalized to the point of driving bilateral clonic seizures, typically referred to as ’stage 5 seizures’ (Racine, 1972). At this point, the initial, brief focal afterdischarge (AD) has been dramatically altered, usually increasing in duration, amplitude, spike frequency, and spike morphology (Racine, 1972). The seizure threshold is also typically reduced over the course of kindling (Racine, 1972). Once generalized kindled seizures (stage 5 seizures) are established, these alterations persist for months or years.

In contrast to kindling, status epilepticus and its sequelae are easier to produce, but more variable in their expression. Most typically, high doses of a convulsant agent such as kainate or pilocarpine are injected systemically. The status epilepticus is characterized by continual,

recurrent seizures. For most researchers, it is not the status epilepticus that is of interest, but the delayed appearance of spontaneous seizures. Thus, the model has been called “post-status epilepticus models of TLE” by Löscher (2002). The morphological changes that occur in the hippocampus following status epilepticus are often quite similar to those seen in human mesial TLE, although the damage in the animal model can be more severe.

Therefore, these models demonstrate that epileptic activity itself can cause lasting alterations in neural function, some of which can lead to permanent epileptogenesis. However, we now know that seizure discharges, whether triggered by electrical stimulation or by a chemical agent, can produce a very large number of post-activation effects, including changes in mRNA and protein for neurotransmitters, receptors, ion channels, intracellular messenger systems, and neurotrophic factors. This massive array of effects complicates the interpretation of data obtained in experimental epilepsy research (Morimoto et al., 2004).

2.1.1. The hippocampus as a frequent site of epileptogenesis

The interest of researchers in the hippocampus is ensured by the fact that it is so highly reactive to a variety of treatments. In animal models, the hippocampus has the lowest seizure threshold of all the structures thus far tested. It is also highly susceptible to both the neuronal damage and circuit reorganization induced by repeated epileptiform discharge. Virtually any substantial elevation over the normal levels of activity in this structure triggers hundreds of measurable changes in hippocampal cells, circuits, and functions. The seizure-induced alterations that have received the most attention recently are the growth of new neurons and new pathways. However, few causal relationships have been established between these alterations and the epileptic state. (Morimoto et al. 2004).

If the hippocampus is critical for the generation of an epileptic response, then its removal should suppress the epileptic response. Most lesion and knife-cut studies, however, indicate that no single structure is critical for the development of a generalized evoked seizure response. In particular, hippocampal lesions produce only a slight retardation in kindling development from other sites (Dasheiff and McNamara, 1982; Sutula et al., 1986; Tanaka et al., 1991) or no effect at all (Racine et al., 1988). Consistent with these results, transient inhibition of the CA1 region of the dorsal hippocampus by focal injection of lidocaine retards amygdala kindling development by approximately 40%, and it slightly reduces generalized seizure duration of amygdala kindled seizures (Mirnajafi-Zadeh et al., 2002). These results suggest that epileptic circuitry may be built up from a variety of alternative pathways.

2.1.2. Hypotheses of the mechanism of epileptogenesis

The term ’hippocampal sclerosis’ refers to morphological changes that include a selective loss of neurons in the dentate hilus and the hippocampal pyramidal-cell layer, with relative preservation of dentate granule cells and a small zone of pyramidal cells in CA2 field of the hippocampus. The dense gliosis that accompanies the loss of neurons causes shrinkage and hardening of tissue. Additionally, dentate granule cells sprout mossy fiber axons that are directed back into the inner molecular layer, possibly because the pyramidal neurons to which they usually extend have been lost. These aberrant mossy fibers may instigate a recurrent excitatory circuit by forming synapsis on the dendrites of neighboring dentate granule cells.

Some investigators have suggested that the selective vulnerability of certain neurons may be a mechanism of epileptogenesis in hippocampal sclerosis. In animal models, excitatory interneurons of the dentate gyrus, which normally activate inhibitory neurons, appear to be selectively lost, and it would be expected to impair the inhibitory feedback and feed-forward mechanisms that act on dentate granule cells, resulting in hyperexcitability.

The differentiation of new granule cells after seizures could be another relevant mechanism of epileptogenesis, since new dentate granule cells become abnormally integrated into neuronal circuits (Morimoto et al., 2004).

2.2. Preconditioning effects of seizures

Stressful and potentially noxious insults that are subthreshold for damage are capable of rendering the brain refractory to damage incurred by a subsequent, prolonged and otherwise harmful stressor. This process, termed preconditioning, is a highly conserved endogenous mechanism by which brain can protect itself (tolerance). The molecular mechanisms underlying this process may yield novel and potent neuroprotective strategies to mitigate the harmful effects of neurological insults such as ischemia, traumatic brain injury and prolonged seizures (status epilepticus).

The first example of seizure activity protecting the brain against further seizure activity was presented by Kelly and McIntyre (1994), where hippocampal kindling protected several structures from the neuronal damage induced by kainate-induced status epilepticus. Kondratyev et al. (2001) also found that repeated electroconvulsive shock (ECS) over a 1-week period protected the brain against the neurodegenerative effects of kainate-induced status epilepticus. In contrast, Andre et al. (2000) compared the effects of prior kindling or ECS seizures on subsequent responses to pilocarpine administration. Prior kindling provided a partial neuroprotection, restricting damage to the entorhinal cortex and the hilum of the

dentate gyrus. Prior ECS seizures, on the other hand, resulted in an increased level of damage following pilocarpine-induced status, particularly in the piriform and entorhinal cortices. Spontaneous seizures developed normally following kindling plus status, but were blocked in the ECS plus status rats. The authors concluded that the increased damage blocked the recruitment of circuitry essential to the seizure event. Penner et al. (2001) reported a significant neuroprotection against kainate-induced damage in rats that had been rapidly kindled from the dorsal hippocampus 21–25 days previously. Bragin et al. (2002) kindled rats from the perforant path following intra-hippocampal administration of kainate. Although the initial AD thresholds were the same in kainate and control groups, the thresholds increased over the course of kindling in the kainate-treated rats. The kainate/kindling groups also showed an increased paired-pulse depression of late components in the evoked response and a decrease in the development of spontaneous seizures. One possible explanation for the neuroprotective effects is that inhibitory output from the primed sites suppresses excitability in the test sites. Ross et al. (2001), however, have published results indicating that some of these suppression mechanisms may survive the removal of the primed sites. They subjected rats to audiogenic seizure priming during development, and then tested area CA1 responses in hippocampal slices from postnatal days 28 to 50. These slices were more resistant to stimulation induced epileptiform discharge. Semyanov et al. (2000) offered a somewhat different interpretation when monitoring an in vitro kindling-like effect in slices from hippocampally- and audiogenically-kindled rats. They found that the in vitro “kindling” effect (a stronger seizure response after the slice had been exposed to repeated K^+ concentration increases) was reduced in the primed animals. The authors saw this as a kind of occlusion phenomenon and argued that the seizure circuitries are likely the same.

3. Transcriptional regulation in the brain

Acute and chronic application of a great variety of stimuli to the brain have been shown to lead to differential responses reflecting short-term and long-term neural plasticity. Thus, the cellular consequences of repeated seizure activity characteristic of epilepsy differ from that of a single, acute seizure episode. This fact is demonstrable by the expression of transcription factors (TFs), proteins that control gene expression in response to receptor activation.

Fos-family TFs exert their effects via heterodimeric complexes that constitute the activator protein -1 (AP-1) complex and activate or repress the transcription of many neurobiologically important target genes (Herdegen and Leah, 1998). While c-fos and full-length FosB are

induced rapidly and transiently in specific brain areas after different types of stimuli, the truncated form of FosB gene, named ΔFosB, codes for a highly stable protein with a half-life of approximately 1 week and functions as a type of 'molecular switch' that gradually converts acute responses into relatively stable adaptations that underlie long-term neural and behavioural plasticity to repeated stimuli (Nestler et al., 1999).

3.1. C-fos expression in seizure

C-fos was one of the first genes shown to be upregulated by activation (Dragunow and Robertson, 1987) and is a sensitive indicator of regions of activity and associated gene regulation. Generally, membrane depolarisation, Ca^{++} influx, cAMP and growth factors are able to influence c-fos expression, predominantly by means of intracellular protein kinase cascades. Seizure-induced c-fos expression is mediated by transmitters acting on ionotropic receptors and voltage-dependent Ca^{++} channels in the membrane (Greenberg and Ziff, 2001).
C-fos and other immediate early genes (IEGs) regulate the expression of neuropeptides and growth factors after brief seizures and may be responsible for the neuronal or nerve terminal sprouting that occur after brief seizures. These effects may be epileptogenic and contribute to the development of epilepsy in the brain. Alternatively, IEGs may induce expression of molecules (e.g., thyrotropin-releasing hormone) that are anticonvulsant and may be responsible for postseizure anticonvulsant effects (Hughes and Dragunow, 1995).

3.2. FosB expression in seizures

Long-lasting induction of specific Fos-family antigens has been shown to accompany several forms of neural and behavioural plasticity related to drug addiction (Hope et al., 1994; Nye and Nestler, 1996; Moratalla et al., 1996), stress responses (Perotti et al., 2003), the clinical actions of psychotherapeutic drugs (Hiroi and Graybiel, 1996), electroconvulsive antidepressant treatment (Hope et al., 1994), certain lesions (Doucet et al., 1996; Mandelzys, 1997) and epileptogenesis (Morris et al., 2000; Madsen et al., 2006).
The investigation of seizures induced by systemically applied kainate using reverse transcriptase–polymerase chain reaction (RT-PCR) technique proved that ΔFosB expression lasted for only 6 h and the persistently expressed 35-kDa Fos-related protein revealed by Western blot analysis was thus considered different from ΔFosB (Bing et al., 1997). However, recent studies demonstrated that unlike the protein, the half-life of ΔFosB mRNA is only slightly longer than that of full-length FosB (Alibhai et al., 2007). These data indicate the possibility that the persistent expression of Fos-related antigens detected after different stimuli

is due to the accumulation of the spliced variant of the FosB gene named ΔFosB. Moreover, in vitro studies confirmed that ΔFosB DNA encodes the expression of every multiple chronic Fos-related antigen (Chen et al., 1997).

FosB expression in basal conditions is related to some neurons scattered throughout layers II, IV, V and VI in the rat cortex, and in the dorsal portion of the dentate gyrus. It is also seen in some areas that express other AP-1 proteins. These findings do not necessarily contradict the reported absence of FosB in murine whole brain homogenate. In the rat visual cortex, basal AP-1 consists mostly of FosB and JunD (Herdegen and Leah, 1998).

4. Changes in inhibitory activity in seizures

'Perhaps the easiest way to trigger a state of runaway excitation is by reducing the levels of inhibition, and failure of inhibition is a widely proposed candidate mechanism for epilepsy' (Morimoto et al. 2004). The importance of gamma aminobutyric acid (GABA) systems in the control of neural activity and the suppression of epileptiform discharge is well established. It is less clear whether long-term changes in the function of inhibitory systems play a role in the development of epilepsy. Though, literature data show, that GABA systems can be modified by seizure activity. Some of these changes may serve a compensatory role, providing increased protection against excess excitation. Other changes however, may promote the epileptic state. GABA is a principal inhibitory neurotransmitter in the mammalian central nervous system, producing inhibitory postsynaptic potentials (IPSPs) in both feedforward and feedback circuits. GABA receptors are generally classified into two subtypes; GABA–A receptors mediate fast IPSPs via Cl^- influx, and GABA–B receptors are believed to function as presynaptic autoreceptors and mediate slow IPSPs via an increased K^+ conductance. In general, selective antagonists of GABA–A receptors are proconvulsants, whereas the agonists are anticonvulsants.

Parvalbumin (PV), a highly diffusible cytosolic Ca^{++}- binding protein, is a cellular marker for fast spiking interneurons in various regions of the rat brain. The discharge characteristics of these interneurons are supposed to be critically dependent on the Ca^{++}-buffering action of PV. PV-containing neurons comprise a characteristic population of GABAergic interneurons in the hippocampus: basket- and axo-axonic cells mediate mainly perisomatic inhibition (Gulyás et al., 1999).

Recently, Sloviter et al. (2003) showed that dentate PV-immunoreactive neurons showed decreased staining but not death after status epilepticus, suggesting the relative resistance of

dentate inhibitory basket cells to activation-induced damage. On the other hand, Cossart et al. (2001) demonstrated that spontaneous GABAergic inhibition was increased in the soma, but reduced in the dendrites of CA1 pyramidal neurons in the pilocarpine model. They suggested that the decreased dendritic inhibition, which may be based on decreased numbers of somatostatin-containing GABAergic interneurons, facilitates the generation and propagation of large dendritic excitatory postsynaptic currents (EPSCs) to the soma, whereas the increased somatic inhibition prevents the occurrence of persistent seizures.

Abnormal intracortical inhibition and facilitation was observed in adult TLE patients during extraoperative cortical stimulation, suggesting a remote effect of epileptic activity onto the motor cortex, that leads to an alteration in local inhibitory circuits. Changes in cortical circuitry and GABA-mediated cortical inhibition have been observed in different epileptogenic lesions. In TLE, abnormal staining for PV, which is expressed by a subpopulation of GABAergic local circuit neurons, was found in neocortical tissue, associated with an increased excitatory and decreased inhibitory synaptic density. These findings are consistent with the hypothesis of a participation of the neocortex in TLE (Silva et al, 2002).

Magloczky and Freund (1993) compared the vulnerabilities of subpopulations of interneurons in the hippocampus following unilateral kainate injection into area CA3. They immunostained for PV, calbindin-D28k, calretinin, and somatostatin, and they found that the hilar somatostatin neurons, the spiny calretinin cells, and the mossy cells were vulnerable, while the other subpopulations were resistant. Morin et al. (1998) used immunocytochemical techniques to show that the density of GAD-positive cells was reduced following kainate treatment, but only in the stratum oriens and the alveus of area CA1. Counts of Nissl-stained neurons were also reduced in this layer 2 weeks after kainate treatment. These results suggest a loss of GABA neurons in the basal dendritic layer of the CA1 region. However, the evidence from the kindling model has been contradictory. For example, although Kamphuis et al. (1987) reported a persistent reduction of GABA immunoreactivity in the CA1 region, it was not replicated in a later study (Lehmann et al., 1996).

5. 4-Aminopyridine as a convulsant agent

4-Aminopyridine (4-AP) is a convulsant that blocks some of the voltage-dependent neuronal potassium channels: the K_A-channel (or A-channel), which regulates the spike frequency in postsynaptic structures, and the K_V-channel (or delayed rectifier), which is involved in the repolarization phase of the action potential, resulting in prolonged action potential duration.

Therefore 4-AP increases the inflow of Ca^{++} into the presynaptic axons (Thesleff, 1980). The drug also acts directly through the presynaptic voltage-sensitive Ca^{++} channels, facilitating transmitter release. The increased presynaptic activity caused by 4-AP is reflected in the increased synaptic vesicle exocytosis at the ultrastructural level. Additionally, 4-AP crosses the blood–brain barrier quickly and will be secreted into the cerebrospinal fluid and eliminated by the kidney. In consequence of its fast action, the latency of the seizure is relatively short, and the convulsive activity probably extends to the whole forebrain. 4-AP is used for seizure induction both in vivo and in vitro and most of its actions are blocked by the standard antiepileptic drugs. The excitatory properties of 4-AP and its derivatives have been examined in humans. Recent studies from our laboratory indicated significant increases in regional cerebral blood flow (rCBF) in the dentate gyrus, neocortex and diencephalon in mice following 4-AP injection (Mihály et al, 2000).

The epileptogenic effects of 4-AP has been extensively studied in acute experiments. The c-fos expression, that can be regarded as a tool to study neuronal activation and seizure spread (Morgan and Curran, 1991), has a characteristic pattern and is maximal at 1–3 h following systemic 4-AP administration in the hippocampus and neocortex (Mihály et al., 2001, 2005). Literature data indicate that the increased transmitter release induced the c-fos expression (Herdegen and Leah, 1998). Microdialysis experiments prove that 4-AP infusion increases the extracellular glutamate concentration in the hippocampus of rats (Pena and Tapia, 2000). However, 4-AP also releases transmitters other than glutamate: the extracellular GABA (Pena and Tapia, 2000), noradrenaline (Versteeg et al., 1995) and dopamine (Bonnano et al., 2000) concentrations increased following 4-AP treatment in vivo and in vitro. It is thought that different glutamate receptors may play a role in c-fos gene expression: the blockade of NMDA receptors inhibited the expression of c-fos mRNA in the dentate fascia (Labiner et al., 1993). Accordingly, previous experiments supported the importance of N-methyl-D-aspartate (NMDA) receptors in the induction of c-fos in 4-AP-elicited seizures (Szakács et al., 2003). The significance of the c-fos expression in the development of seizure activity still remained unclear.

In conclusion, acute seizure experiments using 4-AP revealed alterations in different brain regions involved in the mechanism of seizure generation. However, repeatedly elicited 4-AP seizures are expected to cause qualitatively different cellular modifications as compared to a single seizure episode.

6. Aims

The present study comprises investigations directed towards revealing some features of the mechanisms and consequences of seizure activity in acute and chronic conditions.

The K^+ channel blocker 4-AP was used in order to initiate brief generalized seizures in rats. The differential response after varying number of convulsive episodes was investigated at behavioural and molecular level.

The specific aims of the experiments were as follows:

- Investigation of the relevance of transcriptional regulation in the mechanism of seizure induction and seizure spread by detection of time-dependent c-fos expression.
- Characterization of a chronic seizure model in terms of transcriptional regulation after repeatedly induced seizures that imply long-term alterations in neural plasticity.
- Investigation of the effects of repeatedly induced seizures related to the inhibitory neuronal circuit by examination of changes of a subset of parvalbumin-containing neurons.

MATERIALS AND METHODS

1. Experimental animals and their treatments

Adult rats 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 (Mihály et al., 2001, 2005). Control animals received appropriate volumes of physiological saline. 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.

The investigations were conducted with the following experimental groups:

- 15 Wistar rats were used for the immunodetection of c-fos 30 min, 1 h, 3 h, 5 h, and 8 h after a single injection of 4-AP.
- Male Wistar rats (150–180 g) were anaesthetized with Calypsol (100 mg/kg) plus atropine (0.01 mg/kg) given intraperitoneally (i.p.). The head of the animal was fixed in a stereotaxic frame, and following a vertical skin incision, the soft tissues and the temporalis muscle were cut in order to expose the temporal squama on the left side. The bone was cut with a dental drill, removed and the rhinal sulcus was identified. The cortical area inferior to the rhinal sulcus was electrocoagulated and suctioned (lateral entorhinal cortex ablation, LECA). The lesion of the LEC extended to all cortical layers and also the subcortical white matter, including the temporoammonic pathway. At the end of the procedure, the bone defect was covered with the temporalis muscle and skin was closed with clamps. In sham-operated control (SOC) animals the same procedure was performed except for coagulation – the meninges and the brain were not injured. 40 days following the surgical destruction of the lateral entorhinal cortex (LECA) one group of Wistar rats (3 animals) received a single injection of 4-AP. Three SOC rats received the same treatment. These rats were sacrificed 3 h after the treatment and their brains were processed for c-fos immunohistochemistry (IHC).
- Three groups of Wistar rats (9 animals) received daily 4-AP injections for 1, 4, 8 and 12 days and were sacrificed 24 h after the last injection to be investigated by FosB and PV immunodetection.

- Three groups of Sprague-Dawley rats were treated by daily injections of 4-AP for 12 days and sacrificed 3 h after the last injection to be investigated by PV immunohistochemistry (3 animals), Western blotting (4 animals) and in situ hybridization (5 animals), respectively.

For IHC, animals were deeply anesthetized with diethylether 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 1h. After postfixation, the brains were cryoprotected overnight in 30% sucrose in 0.1 M phosphate buffer (pH 7.4).

2. Immunohistochemistry

2.1. FosB immunolabeling

Sections were incubated in 0.2% Triton X-100/PBS for 20 min, followed by washing in three changes of PBS. Then 20% normal pig serum (NPS; Sigma) was applied for 1 h to block nonspecific labeling. Sections from rats injected for 1, 4 and 8 days were used for immunohistochemical analysis of FosB protein, while sections from the rats treated for 12 days were used for simple FosB immunolabeling. Since antibodies selective for ΔFosB are not available, we used a rabbit polyclonal antiserum raised against an internal region of FosB (sc-48; Santa Cruz Biotechnology), that was shown to recognize both FosB and ΔFosB (Perotti et al., 2005). The antibody was used diluted 1:4000 in 0.05M PBS containing 10% NPS. The sections were incubated in the primary antibody overnight at room temperature (RT).

The biotinylated secondary antibody (goat anti-rabbit IgG; Vector Laboratories, CA) was diluted 1:40 in PBS, and sections were incubated for 1 h. After additional washing, sections were transferred to streptavidin-peroxidase (1:1000; Jackson ImmunoResearch), and incubated for 1 h. Then the sections were washed and processed using 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma) containing 0.3% nickel sulphate and 0.01% hydrogen peroxide. After 20 min of incubation, the chromogen yielded a black reaction product.

The sections were dehydrated with an ascending series of ethanol (40%, 70% and 100%), cleared in xylene and mounted with Entellan (Fluka).

2.2. C-fos immunolabeling

Frozen, coronal plane sections were cut at 20 μm thickness with cryostat. Polyclonal c-fos antibody (raised in rabbit; Santa Cruz Biotechnology, CA, USA) and the peroxidase-antiperoxidase (PAP) method was used for immunohistochemistry. The sections were incubated in 20% normal pig serum, next in primary c-fos antibody (1:1000 in 20% normal pig serum), and then in donkey antirabbit IgG (1:40; Jackson Immuno-Research, PA, USA). The secondary antibody was detected by to peroxidase-anti-peroxidase (PAP) complex diluted to 1:1000 (Jackson). The peroxidase reaction was localised with nickel chloride-containing DAB (Sigma), yielding a black reaction product.

2.3. PV immunolabeling

Coronal plane frozen brain sections (24 μm thin) were reacted with monoclonal mouse anti-PV antibody (Sigma PA-235, dilution 1:15000). 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 DAB (Sigma) as a chromogen. The sections were mounted on microscope slides, dehydrated and coverslipped with Entellan (Fluka).

2.4. PV and FosB double immunolabeling

Double immunolabeling was performed using primary antibody cocktails: mouse anti-PV (Sigma), diluted 1:100,000 and rabbit anti FosB (Santa Cruz Biotechnology), diluted 1:500. The sections were incubated in the primary antibody cocktail overnight at RT, then transferred to the secondary antibody cocktail: biotinylated anti-mouse IgG (Sigma), diluted 1:600, and unlabeled goat anti-rabbit IgG (Vector Laboratories) diluted 1:40. The sections were incubated for 1 h in the secondary antibody cocktail and then transferred to streptavidin-peroxidase (1:1000; Jackson ImmunoResearch). The streptavidin-peroxidase was developed by using 0.05% DAB (Sigma) with 0.01% hydrogen peroxidase. Following a wash in TBS, the sections were transferred to PAP (Jackson ImmunoResearch), diluted 1:1000, and incubated for 1 h. The PAP was developed using 0.05% DAB (Sigma) with 0.3% nickel sulphate and 0.01% hydrogen peroxide, as described previously.

3. Evaluation of immunolabeling and statistical analysis

The quantitative analysis of each immunolabeling was performed on five histological sections per animal. The immunoreactive cells were counted using a Nikon Eclipse 600 microscope equipped with a Spot RT Slider digital camera (1600x1200 dpi in 8 bits), and the Image Pro Plus 4 morphometry software (Media Cybernetics, Silver Spring, MD).

Areas of interest were selected on the basis of the same stereotaxic coordinates (Paxinos and Watson, 1998): from regions CA1, CA2, CA3 of the Ammon's horn, the granule cell layer of the dentate gyrus, the hilum of the dentate gyrus, and from the neocortex.

In the neocortex, the area of interest was the rectangular image-capturing field of the camera which included all neocortical layers (I–VI) from the pia mater to the subcortical white matter. Frozen sections stained with cresyl violet were used as reference for the thickness of the neocortical layers. The counting was done by using a 10x objective. Following background subtraction, the threshold was adjusted so that all positively labeled cells could be recognized. Cell counts were then normalized to 1 mm^2.

In the hippocampus, the area of interest was the rectangular image-capturing field of the camera. The hilum of the dentate fascia was outlined manually, according to Amaral (1978), and used as area of interest. The counting was done by using a 40x objective. Following background subtraction, the threshold was adjusted so that all positively labeled cells could be recognized. Cell counts were then normalized to 1 mm^2.

In the mammillary bodies, the areas of interest (AOI) for cell counts were selected from the medial mammillary nucleus (MM). 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 40x objective. The AOI was the rectangular image-capturing field of the camera (222 μm x 296 μm).

The statistical analysis was performed with the SPSS 9.0 computer program.

Differences of FosB, parvalbumin and double immunolabeling between control and 4-AP treated groups were analysed by a paired t-test in case of each time-course experiment. A significance criterion of $p < 0.05$ was used.

Differences in the number of c-fos IR cells in the control and convulsing animals were analysed with one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test.

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 2mM EDTA. Protein concentrations were determined according to the method of Lowry et al. (1951). 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 5x5 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 x area) using the Gel-Doc system (Biorad). For quantitative comparisons, each value was calculated based on at least seven independent determinations.

ANOVA followed by post hoc analysis (Tukey's multiple comparison) was used to evaluate the significance.

5. cRNA probes

The only PV gene in the rat contains five exons and codes for two mRNA species by means of alternative polyadenylation. A template for a gene-specific cRNA probe was created by isolating the 133 bp long third exon of the rat PV gene by genomic PCR using the primers 5' -CCCCCCAACAGCTGCAGACTCC- 3' and 5'-TTTACCCCAGCTCATCCTCCTCAATG-3'. The PCR product was inserted into the pcDNA3 vector (Invitrogene, Carlsbad, CA)

between BamHI and EcoRI restriction sites and cloned using standard methods. 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 radiolabeling, [^{35}S]UTPα´ S (ICN Biomedicals; Costa Mesa, CA) was incorporated during the syntheses of the cRNA probes. The antisense and sense PV [^{35}S]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 $3.1x10^7$–$6.4x10^7$ cpm/ pmol by liquid scintillation counting.

6. In situ hybridization

Coronal rat brain sections were fixed for 5 min in 2x SSC (NaCl + Sodium citrate) containing 4% formaldehyde at RT and washed twice in 2x 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, 6x SSPE (1xSSPE: 0.18 M NaCl, 10 mM $NaPO_4$ and 1 mM EDTA, pH 7.7), 5x Denhardt's reagent, 10% dextran sulfate, 50 mM dithiothreitol (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 2x SSC/50% formamide at RT for 5 min, twice in 2x SSC/50% formamide at 50 °C for 10 min, and then in 2x SSC at RT for 5 min. The sections were next incubated in 1x TE containing 0.5 M NaCl and $1.32x10^{-3}$ Kunitz U (~25 mg protein)/ml RNase A at 37 °C for 30 min, and rinsed in 2x SSC/50% formamide at 50 °C for 10 min and in 2x SSC at 50 °C for 10 min. Sections were dehydrated, air-dried and processed for phosphorimaging.

Hybridized rat brain sections and membrane standard scales (Palfi et al., 1998), previously calibrated to brain paste standard scales (Vizi et al., 2001), 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 (Vizi et al., 2000). Phosphorimages were captured with Cyclone Storage Phosphor System (Packard) at 600x600

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. Labeling 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 (Vizi et al., 2000). Labeling values of brain areas (net dlu/mm^2) were converted to membrane labeling values (net dlu/mm^2) using a previous calibration (Vizi at al., 2001). Further conversion steps can be summarized in the equation: RES = (LAB x AVO)/(LRF x RDF x SPA), where RES is the result (ISH copy number/mm^2), LAB is the labeling intensity (net dlu/mm^2), AVO is the Avogadro number ($6.02252x10^{23}$ copy number/mol), LRF is the labeling 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 labeling 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 number/mm^2 value of the PV mRNA.

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 test ($p<0.01$ or 0.001), ANOVA followed by a post hoc Bonferroni test ($p<0.05$), and Median test ($p<0.05$).

RESULTS

1. 4-AP-precipitated seizure behaviour

1.1. Acute seizure model

Following the injection of the convulsive dose of 4-AP, the animals displayed behavioural seizures which lasted for 60–80 min. The characteristic symptoms occured within approximately 10 min of injection and developed gradually, as described previously (Mihály et al., 1990). Initially, animals exhibited increased exploratory activity which was followed by tremors of the vibrissal muscles, shivering, forelimb clonus with increasing frequency conducting to the development of generalized tonic-clonic seizure with loss of postural control. After a postictal period of 10–20 min, some of the animals displayed generalized seizure again.

The seizure symptoms were registered according to a scale established by Racine (1972): 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. The average latency of the first GTCS was 30.3 min (±1.4 min).

1.2. Chronic seizure model

During the 12 days' treatment the above-mentioned seizure symptoms were detected every day, although the severity and the stages showed slight variations (Fig. 1).

The analysis of the daily elicited seizure symptoms according to the Racine scale proved that the convulsion pattern changed slightly towards the end of the experiment: decreases of the seizure intensity were observed, i.e. after 4-AP administration the animals developed a single full motor seizure (stage 5) instead of two seizures, or displayed less severe, stage 4, symptoms.

1.3. Lateral entorhinal cortex ablation (LECA)

In the LECA group (n = 7) only three animals responded to 4-AP injection with a single seizure and the remaining four animals produced no seizure at all. In the SOC group the first seizure lasted for 55.5 ± 4.2 s, and developed during the first 30 min following the 4-AP injection with an average latency of 20.8 ± 6.4 min. These events were followed by a second seizure within the first hour following the 4-AP treatment. The mean delay between the two

seizures was 16.2 ± 4.9 min. The second seizure was longer, lasted for 73.4 ± 13.9 s. In the LECA group the latency of first seizure increased to 31.8 ± 4.3 min, and only one rat presented a second epileptic event.

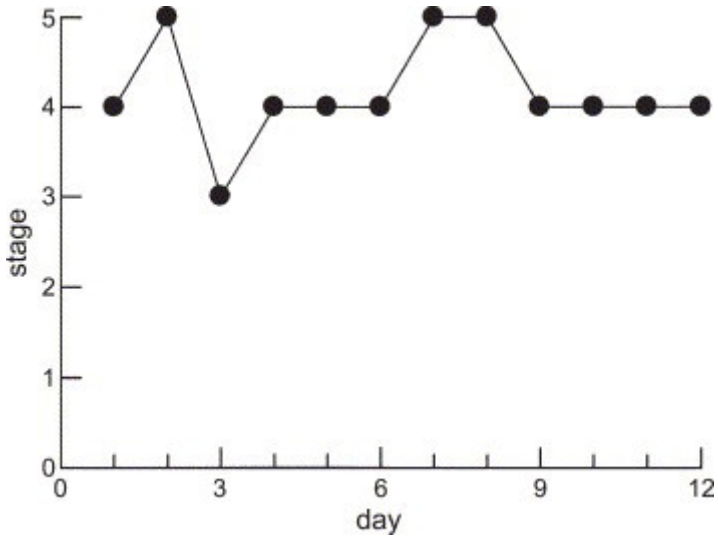

Fig. 1. Seizure symptoms according to Racine, observed in one of the experimental animals during the 12 days of the 4-AP treatment. Stage 1: mouth and facial movements; stage 2: head nodding; stage 3: forelimb clonus; stage 4: rearing; stage 5: full motor seizure (rearing and falling with loss of postural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

2. Immunohistochemical results

2.1. Seizure induced c-fos

The c-fos IR cell nuclei were present in every layer of the somatosensory neocortex following 4-AP administration. The increase of the number of c-fosIR cell nuclei was significant already at 30 min in layers II, III, IV, V and VI. The highest values were detected at 1 h in each layer. The increase was still significant at 3 h following 4-AP injection. Cell counts decreased to control levels at 5 and 8 h following 4-AP administration. The highest elevation in the number of c-fosIR cells at 30 min was seen in layer IV: other layers contained less c-fosIR cells. At 1 h, every layer (except layer I) stained strongly; only layer V stood out with a lower amount of

c-fos IR cells. At 3 h, the number of c-fos IR cells decreased to control levels, and remained so at 8 h (Fig 2.). The values in convulsing animals displayed highly significant differences ($P \leq 0.01$) compared to the controls. In controls, scattered c-fosIR cells were seen in layer IV.

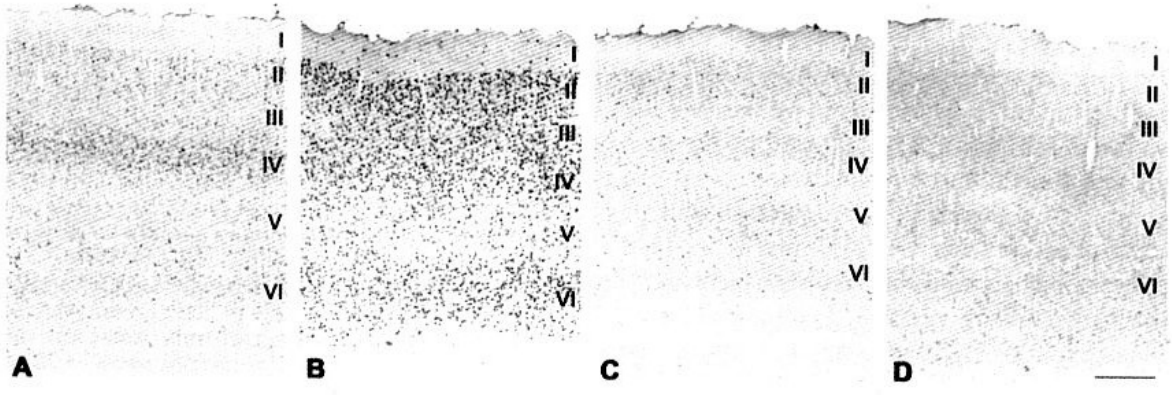

Fig.2. Immunohistochemistry of c-fos protein in the neocortex in 4-AP injected animals (A, 30 min; B, 1 h; C 3 h; D, 5 h). Controls are not shown because they display a staining pattern similar to D. Roman numerals indicate neocortical layers; bar, 500 μm.

2.2. Seizure induced c-fos after LECA

Convulsions induced by 4-AP 40 days after the surgery caused the expression of c-fos protein in every part of the hippocampus and the subiculum. The overall pattern of c-fosIR in SOC and LECA animals 3 h after the 4-AP injection was similar to our previous findings obtained with intact animals (Mihály et al. 2001, 2005; Szakács et al. 2003): strong c-fosIR in the dentate granule cell layer, medium c-fosIR in the stratum pyramidale of CA1–3, and scattered c-fosIR nuclei in the hilum of the dentate gyrus, and in the infra- and suprapyramidal layers of CA1–3. Quantitative analysis of immunostained cell nuclei revealed that the convulsions in LECA animals induced significantly less c-fos immunopositive cells in the pyramidal cell layer of the Ammon's horn, and in the granule cell layer and in the hilum of the dentate gyrus, compared with the SOC group. Because the number of the haematoxylin-eosin-stained cell bodies did not change in LECA brains, the significant reduction in the number of c-fos immunopositive cells indicates the decrease of neuronal activity, and not the loss of neurones in the investigated hippocampal regions.

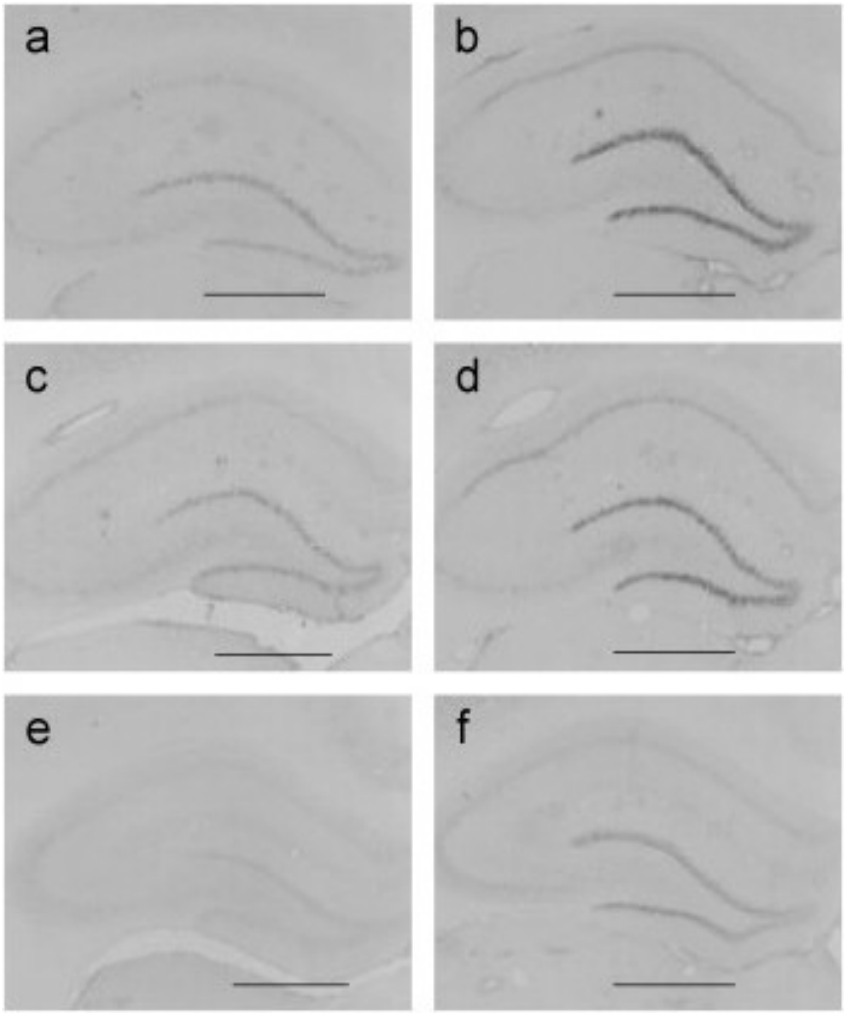

Fig. 3. Low magnification photomicrographs illustrating the distribution of FosB immunoreactive cell nuclei in the hippocampus of 4-AP treated (b, d, f) and control (a, c, e) rats after 4 days (a, b), 8 days (c, d) and 12 days (e, f) of treatment. Scale bar: 1 mm.

2.3. FosB

The FosB immunohistochemistry revealed weak labeling in the Ammon's horn of control and 4-AP treated rats in every time-course group. A small number of weakly labeled cell nuclei were found in this hippocampal region and it was therefore not evaluated by statistical analysis. The dentate gyrus was characterized by a more striking FosB immunolabeling (Figure 3). There was a significant increase in FosB-immunopositivity in the 4-AP treated animals compared with controls after 4, 8 and 12 days of treatment. Increase in immunopositivity after a single 4-AP treatment did not reach statisitical significance in comparison to the corresponding control level (Figure 4a). Neocortical FosB immunoreactivity increased significantly after 4-AP elicited convulsions at every time point

compared to controls (Figure 4b). Sections stained with cresyl violet did not show gross cell loss.

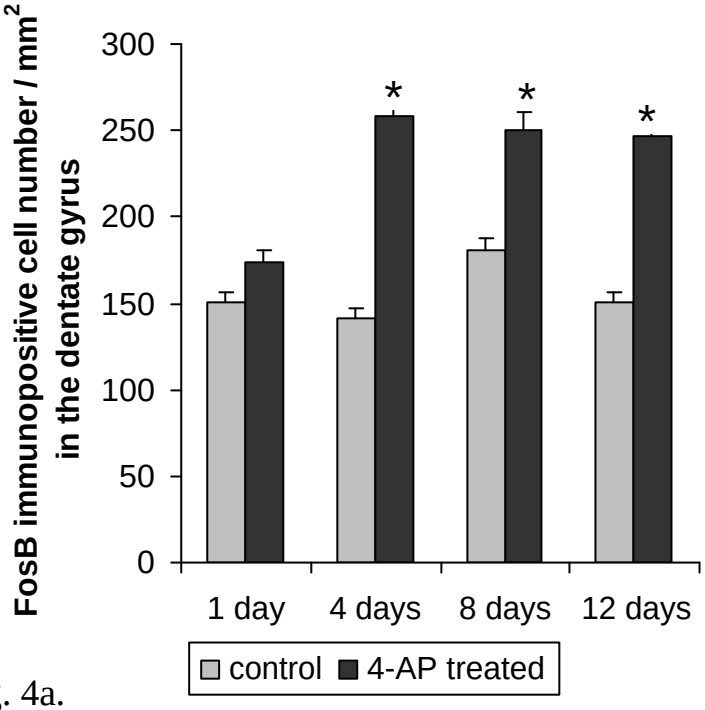

Fig. 4a.

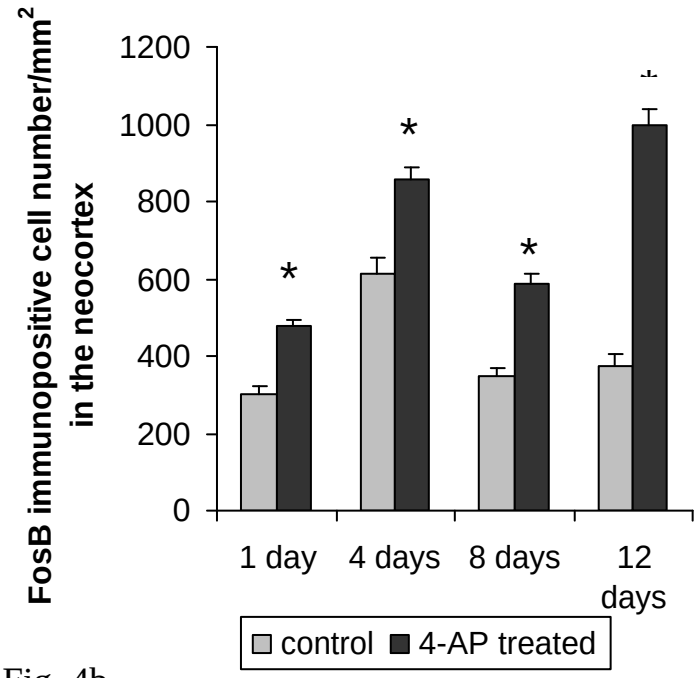

Fig. 4b.

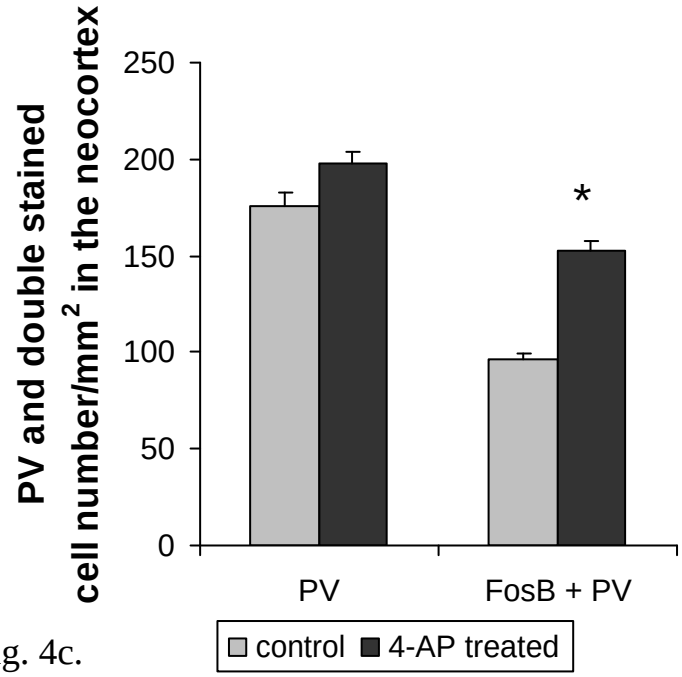
PV and double stained cell number/mm2 in the neocortex
250
200
150
100
50
0
*
PV
FosB + PV
control
4-AP treated

Fig. 4c.

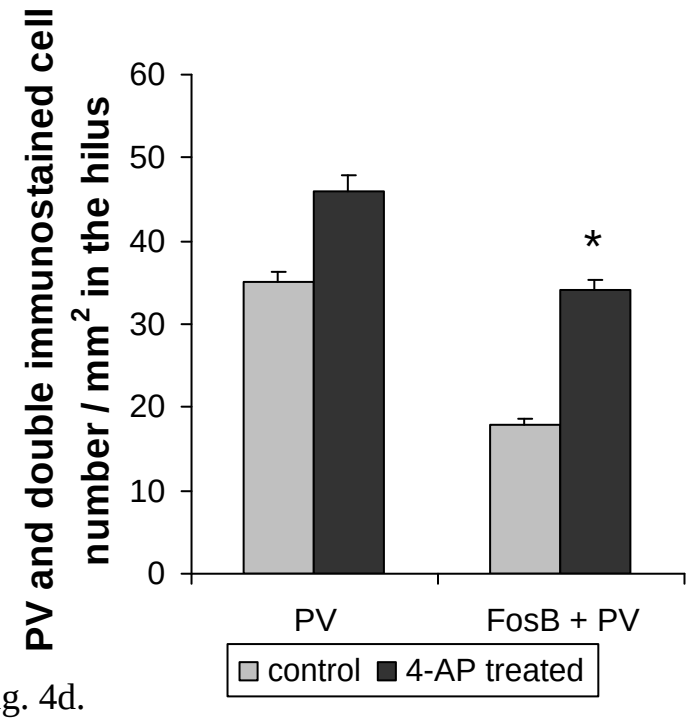
PV and double immunostained cell number / mm2 in the hilus
60
50
40
30
20
10
0
*
PV
FosB + PV
control
4-AP treated

Fig. 4d.

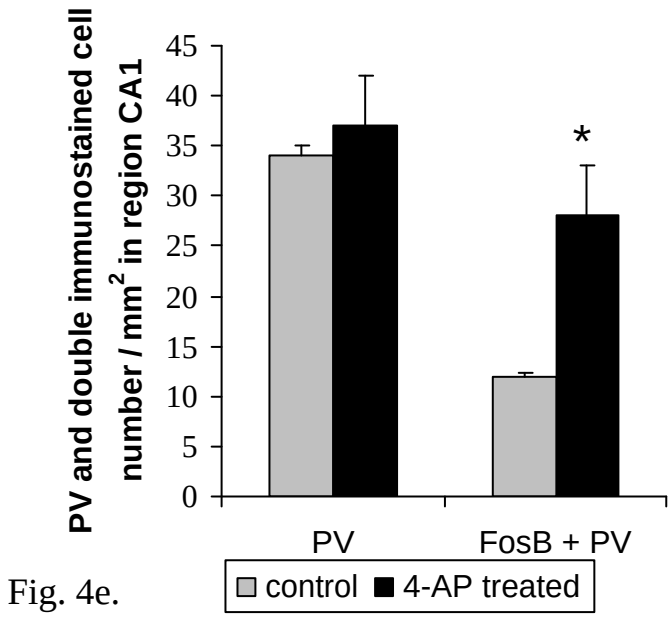

Fig. 4e.

Fig. 4. Results of FosB immunopositive cell counts in the dentate gyrus (a) and frontal cortex (b) of 4-AP injected and control groups after 1, 4, 8 and 12 days of treatment, and results of the counting of FosB and FosB+PV double immunopositive neurons in the frontal cortex (c), hilum of the dentate gyrus (d) and CA1 (e) in 4-AP treated and control groups after 12 days of treatment. Asterisks denote significant differences ($p < 0.05$). Standard error of means (S.E.M.) is indicated on the top of the column.

2.4. PV in the medial mammillary nucleus

The PV immunoreactivity in the MM (Fig. 5) was similar to that described (Celio, 1990). Medium-sized neurons with few dendritic processes contained PV-like staining. Regional densities of immunolabeled 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. 5), although the intensity of the staining did show a visible decrease. No pathological cell forms were detected in the 12 days' 4-AP samples.

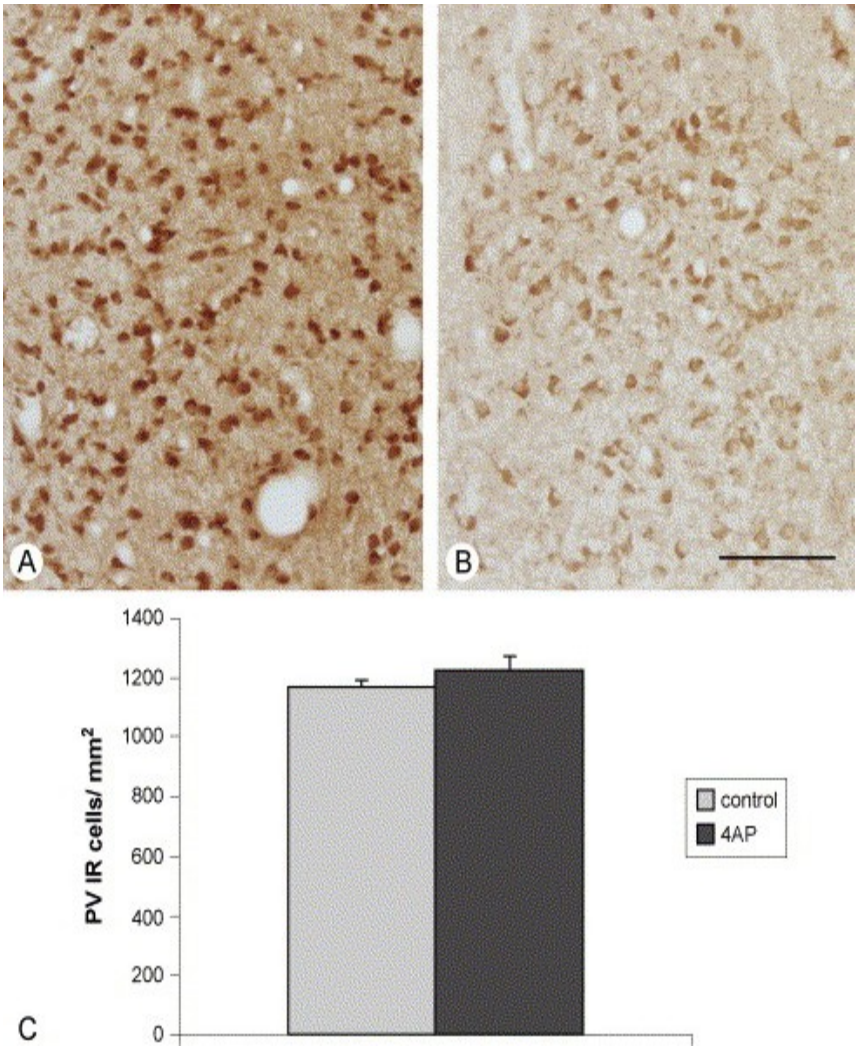

Fig. 5. PV immunopositive neurons in MM following 12 days saline (A) and 4-AP (B) treatment. The number of PV immunopositive cells in MM were quantified and compared to corresponding control values (C). No changes were detected. Note that the intensity of immunostaining is lower in convulsing animals. Scale bar 100 μm.

2.5. PV and FosB in the cortex

In the brain sections from animals treated for 12 days from both 4-AP injected and control animals, PV-positive neurons were distributed in every layer of the frontal cortex (Fig. 6). In the hippocampus, PV-positive neurons were present mainly in the pyramidal layer of the Ammon's horn and in the hilar region, while the granule cell layer displayed scattered PV-immunopositive cells. No significant changes were revealed in the overall number of PV-

immunopositive cells in any of the investigated brain regions after 4-AP treatment. However, there was a significant increase in double-labeled neurons after 4-AP treatment in comparison to controls in the neocortex, hilum of the dentate gyrus and region CA1 (Fig. 4 c, d, e).

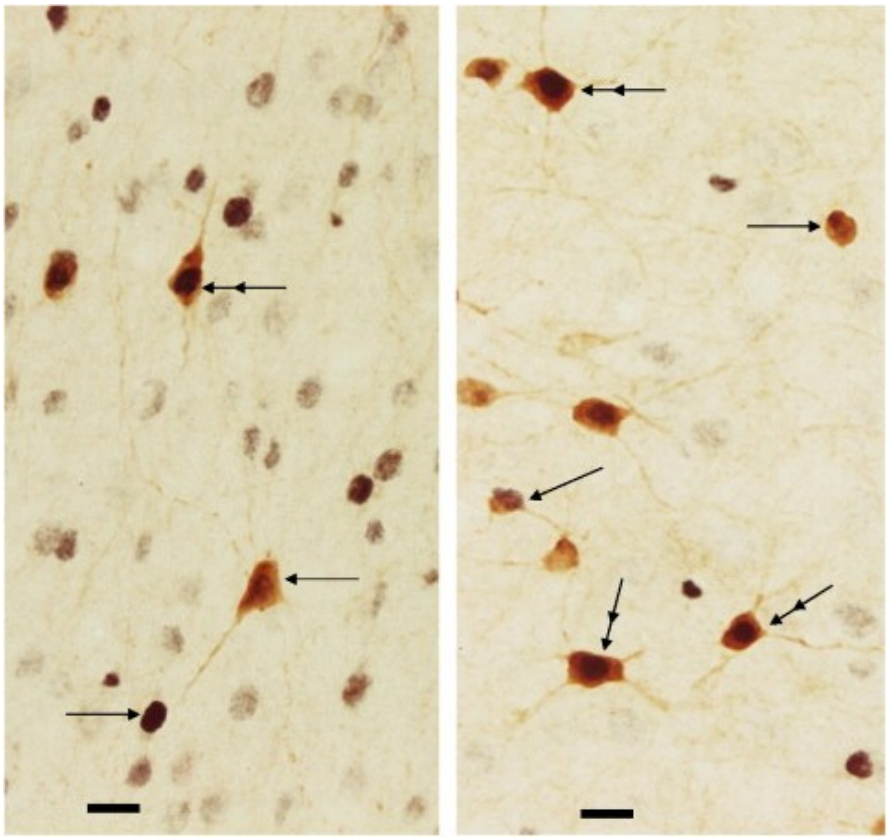

Fig. 6. Appearance of the PV+FosB double immunolabelling in the neocortex. Double arrows point to the double-labelled cells, while simple arrows indicate PV-positive FosB-negative neurons and PV-negative FosB-positive cells. Scale bar: 10 μm.

3. Western blotting of the mammillary area

PV immunoreactive bands were sharp. The samples from control and 4-AP-treated rats displayed a visible density difference (Fig. 7). 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 difference between control and 4-AP-treated values was significant ($p < 0.05$).

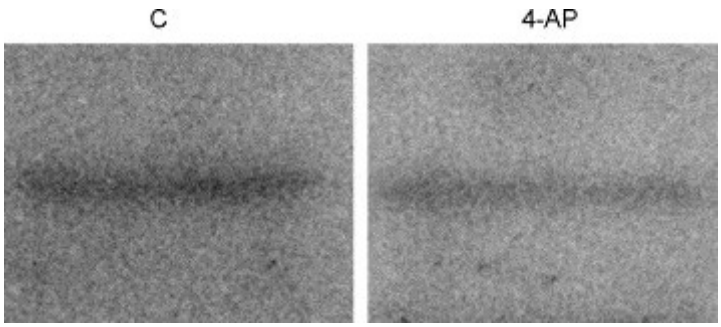

Fig. 7. Western blots from 12 days control (C) and 12 days 4-AP-treated (4-AP) animals. The ECL method displays visible differences between the two bands. Density analysis of the blots revealed a 20.1% significant ($p < 0.05$) difference, i.e. a 20.1% decrease of PV content in the medial mammillary area in convulsing animals.

4. 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 [^{35}S]cRNA probes. Autoradiograms of sections hybridized with antisense PV [35S]cRNA probes displayed a characteristic labeling pattern specific for PV mRNAs (Fig. 8A–H), (Seto-Ohshima et al., 1989). Images of sections hybridized with sense PV [^{35}S]cRNAs had very low level of labeling and did not exhibit brain structure specific signals (Fig. 8I). 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 (Allen et al., 1988) of the medial mammillary nucleus (MM) could not be separated on the ISH images (Fig. 8). 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. 8).

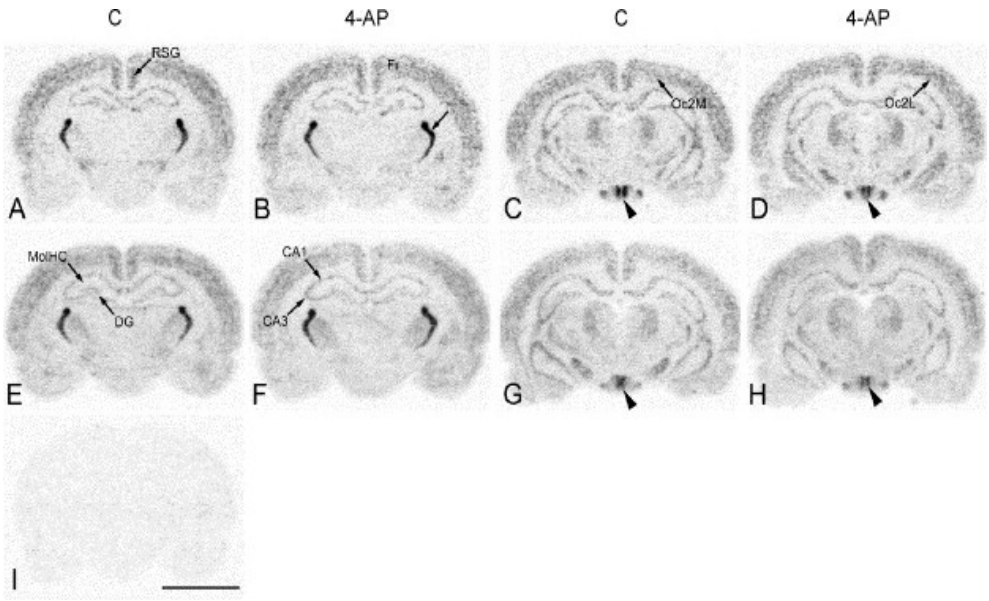

Fig. 8. Expression of PV mRNAs in the rat brain following acute (3 h: A–D) and chronic (12 days: E–H) 4-AP treatment. Coronal sections cut at bregma −3.14 and at bregma −4.52 were hybridized with antisense (A–H) and sense (I) PV [^{35}S]cRNA probes. Arrowheads indicate the medial mammillary nucleus displaying 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.

Table 1. Regional abundances of PV mRNAs in the rat brain after acute and chronic 4-AP treatments. The presented values are ISH copy numbers ($x10^3$)±S.D. corresponding to 1 mm^2 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.

Cerebral neocortex (layers 2–6)		
Frontal	91.1±16.4	102.8±19.0
	89.8±11.9	110.9±32.9
Hindlimb	124.8±18.4	125.5±17.8
	120.7±13.7	140.0±32.9
Parietal b	155.3±14.8	181.3±27.9
	166.2±21.5	194.3±38.1
Parietal c	117.1±16.8	147.8±21.2
	120.9±14.4	143.7±16.4
Occipital 2M	101.3±19.1	108.6±16.0
	86.7±4.9	117.1±17.7
Occipital 2L	136.4±20.1	134.5±23.3
	129.4±20.1	147.4±32.8
Temporal 1	165.0±13.7	162.0±21.6
	152.3±6.3	166.9±27.1
CA1 pyramidal cell layer,	142.4±28.9	142.6±32.4
hippocampus	122.7±38.8	163.1±39.9
CA3 pyramidal cell layer,	131.7±37.8	184.4±65.4
hippocampus	149.0±40.9	165.8±56.0
Granule cell layer,	120.7±22.0	119.6±16.3
dentate gyrus	100.3±17.4	135.8±39.7
Medial mammillary	766.6±93.4	668.4±125.8
nucleus	682.3±109.2	514.5±144.5 ↓
Lateral mammillary	315.3±86.6	267.1±78.7
nucleus	324.6±99.1	344.0±108.6
Reticular thalamic	862.7±113.1	929.3±86.5
nucleus	998.6±92.2	953.5±128.8

DISCUSSION

1. C-fos expression in 4-AP elicited seizure

In the acute 4-AP seizure model, one dose of intraperitoneally applied 4-AP induced at least two seizure events in the animals. These brief, repetitive convulsions differed in duration. EEG studies revealed that the duration of the second seizure was significantly longer than that of the first, indicating the sensitization of large neuronal populations following the first convulsion (Mihály et al., 2005). As concerns the c-fos expression in the neocortex, the effects of the two repetitive seizure episodes were different. The first seizure did not generate an increase in c-fos mRNA level, as measured at the 30 min RT-PCR sampling time. The second seizure boosted the transcription of c-fos, causing a highly significant elevation at 1 h, as shown by the RT-PCR measurements. (Mihály et al., 2005).

The immunohistochemical results described in the present study revealed that the number of c-fos IR cell nuclei was significantly elevated already at 30 min, indicating that a large amount of Fos-protein entered the neuronal cell nuclei at the time when c-fos mRNA levels were not yet elevated. This discrepancy raises the possibility of the seizure-activated translocation of the Fos-protein from the cytoplasm to the nucleus. Literature data indicate that the intranuclear translocation of the Fos-protein depends on extracellular signals (Roux et al, 1990). Our observations support these data and suggest that neuronal depolarization and the rise of intracellular Ca^{++} level stimulate the translocation of the c-fos protein from the cytoplasm to the cell nucleus.

The analysis of the different neocortical layers revealed that lamina IV displayed the most c-fos IR cells at 30 min, indicating that the activation of the neocortex probably happened through the thalamocortical projections (Freund et al., 1989). This layer retained a relatively high number of stained cells after 3 h, suggesting that the thalamocorticothalamic circuits are important in the maintanance of synchronized neocortical activity (Steriade and Contreras, 1995). Previous rCBF measurements proved the large increase of rCBF in the dorsal diencephalon and the neocortex in 4-AP seizures in mice (Mihály et al., 2000). Following this line of evidence, we conclude that in acute neocortical seizure the first cellular event is the stimulated translocation of cytoplasmic c-fos protein to the nucleus, where it binds to the AP-1 sequence, and can participate in transactivation processes. The second EEG seizure

event caused the accumulation of the molecular activators of the c-fos promoter, and the large induction of c-fos transcription, as shown by the 1h peak of c-fos mRNA. This process could be the result of the strong activation of Ca^{++}- permeable glutamate receptors in consequence of seizure-induced intracortical pyramidal-pyramidal cell interactions (Thomson and Deutchard, 1994). At 3 h, both c-fos mRNA and protein decreased, indicating the decrease of the transcription and the slow enzymatic breakdown of the synthesized c-fos protein.

The importance of the lateral entorhinal cortex for seizure spread is demonstrated by the altered hippocampal c-fos expression after 4-AP injection. The decrease of hippocampal c-fos expression in LECA animals can be explained by the decrease of the number of brief seizures. Our experiments with 4-AP proved that c-fos mRNA expression is boosted regularly by the second and third brief convulsion (Mihály et al., 2005). Following LECA, animals had only one brief hippocampal convulsion, despite the fact that the convulsive agent 4-AP affected the hippocampus through the circulation.

2. General evaluation of FosB immunodetection

We analysed the effects of daily intraperitoneal 4-AP injections on the immunodistribution of FosB proteins in the hippocampus and frontal cortex and observed seizure behaviour. A FosB antiserum that recognizes both FosB and isoforms of ΔFosB proteins was employed. It has been shown that full-length FosB is transiently induced after stimulation and returns to basal levels within 8–12 h, while two forms of ΔFosB have been identified as having longer half-lives: the 35 kDa protein with an estimated half-life of 28 h, and the 37 kDa protein with an estimated half-life of 208 h (Chen et al., 1997). In order to detect the immunodistribution of the highly stable FosB gene products we sacrificed the experimental animals 24 h after the last injection. Thus, most of the FosB-like immunoreactivity would be due to long-lasting forms of FosB proteins.

Repeated intraperitoneal injections of 4-AP are thought to cause stress-related changes in the brain which must be distinguished from the consequences of the seizure episodes. It has been demonstrated that after repeated stressful experiences, ΔFosB expression increases in some distinct brain areas according to the type of the stressor (Perotti et al., 2003; Conversi et al., 2006). This is why we compared the results of 4-AP treated group and the control group (which also received daily intraperitoneal injections) separately for each time-course experiment.

The pattern of immunolocalization reflected differential adaptation of the investigated brain regions in the four time-course groups. Following a single injection of 4-AP, only the frontal cortex displayed significant increase of FosB-like immunoreactivity. Recently, it has been suggested that ΔFosB mRNA is formed by alternative splicing of the full-length FosB mRNA and this splicing of FosB pre-mRNA is regulated by the quantity of unspliced transcript available to the splicing machinery (Alibhai et al., 2007). Thus, a first stimulus may induce a FosB level high enough for the appearance of a significant amount of ΔFosB that could accumulate after further applied stimuli.

Acute 4-AP seizure is known to result in an intense expression of c-fos that returns to control level at 8 h after the stimulus (Mihály et al., 2005). We expect that c-fos is gradually replaced by FosB, as literature data refer to the reciprocal repression at the promoter region of these genes (Herdegen and Leah, 1998). It is therefore plausible that the pattern of c-fos induction is predicting a certain pattern of FosB expression after a single stimulus.

3. FosB immunolocalization in the frontal cortex and the behavioural pattern of the chronic 4-AP seizures

The FosB immunopositivity in the frontal cortex was increased after 4-AP convulsions at every time point in the experiments reported here. The qualitative changes that are denoted by long-term FosB expression should be considered in relation to the transcriptional regulation of target genes. According to the literature, glutamate receptor subunit expression has been identified as belonging to FosB-regulated gene products. The rearrangement of the distribution of NMDA receptor subunits including the upregulation of NMDAR1 with decreased NMDA-induced inward currents in pyramidal neurons and a parallel desensitization of motor seizure after repeated electroconvulsive seizures have been shown to be associated with the expression of FosB in the frontal cortex (Hiroi et al., 1998). The authors of the study found that repeated administration of ECS induced a progressively shorter motor seizure in rats, an effect that was near maximal by day 3. This form of tolerance is consistent with the clear tolerance that develops in human undergoing chronic treatment with ECS. The FosB mutant mice showed a significant delay in the development of tolerance to motor seizures, even though the motor seizure induced in the mutant mice by the first ECS was indistinguishable from that exhibited by wild-type littermates. Continued treatment with ECS, however, did eventually result in a significant reduction in seizure duration, with

equivalent motor seizures observed in mutant and wild-type mice by day 6 (Hiroi et al., 1998).

The intense immunolabeling of FosB in the frontal cortex may also be correlated with a gradual reduction in the severity of seizure behaviour in our chronic 4-AP experiments. In the chronically treated groups, we did observe a gradual reduction in severity of the motor seizure symptoms evaluated by the Racine scale, the desensitisation occuring towards the third and fourth day. Such a correlation could be further clarified by future studies of changes in NMDA receptor ditribution after 4-AP seizures.

After several days of less severe symptoms, the daily 4-AP treatment led to a further sensitization that caused stage 5 symptoms again. This phenomenon is apparently similar to a kindling effect and may be mediated by a prolonged expression of ΔFosB isoform, considering the possible changes in the transcrptional regulation related to the duration and level of ΔFosB expression (McClung et al., 2004).

Following the short sensitization period of 1-2 days, in the last several days of the 12 days' treatment seizure tolerance was again observed.

In conclusion, the behavioural effects of the daily 4-AP convulsions reflect changes in the excitability throughout the 12 days of treatment, and it appears reasonable to account these changes to the rearrangement of glutamate receptors, some of them being recognized as putative targets of ΔFosB (McClung et al., 2004).

4. FosB immunolocalization in the hippocampus

The hippocampal immunolocalization of FosB reflects differences between the Ammon's horn and dentate gyrus after repeated 4-AP convulsions. The regions of the Ammon's horn displayed a very weak FosB-like immunoreactivity compared to the dentate gyrus. This may also be indicative of an insignificant level of the adaptive changes of NMDA receptors, similar to those observed in the frontal cortex and a consequent vulnerability to the excitotoxicity of glutamate. This is in accordance with a reported vulnerability of the hippocampal regions CA1 and CA3 to infusion of convulsive dose of 4-AP in the hippocampus where the resulting excitotoxic neuronal damage has been shown to be mediated by NMDA receptor activation (Pena and Tapia, 2000).

Changes in immunoreactivity in the dentate gyrus were not statistically significant following the first day of seizure. However, the chronic treatment resulted in significantly increased FosB-like immunoreactivity in this hippocampal region. Moreover, it has been demonstrated

that seizure preconditioning induced a much broader transcriptional response in the dentate granule cell layer than in the pyramidal cell layers of the hippocampus, and activated a series of neuroprotective genes in the dentate gyrus that are induced at a significantly lower level in the Ammon's horn (Borges et al., 2007). Many of these genes implicated in neuronal growth and regeneration are known to be regulated by ΔFosB (Miura et al., 2005). Repeated seizures are known to induce neurogenesis of dentate granule cells (Scharfman et al., 2002).

These issues seem to reflect a neuroprotective effect of persistent presence of FosB that is characteristic of the dentate gyrus rather than of the hippocampus proper. However, the function of the FosB protein is believed to be more complex, as it has a region-specific role in the regulation of different target genes in distinct cell populations that are still not entirely characterized by the existing literature. Furthermore, it is known that depending on the level of FosB protein or the length of time that it is expressed, it can activate or repress gene transcription (McClung et al., 2004). In vivo and in vitro studies revealed a large number of target genes for ΔFosB, some of them in association with apoptosis (Tahara et al., 2003) and proliferation (Miura et al., 2005). Related to injury events, ΔFosB has been demonstrated to have long-lasting elevated levels in the hippocampus after pilocarpine-induced status epilepticus (SE) followed by recurrent spontaneous seizures, suggesting that this TF may play a role in epileptogenesis (Morris et al., 2000). The increased immunoreactivity of FosB, including in the Ammon's horn, 8 weeks after pilocarpine-induced SE may reflect the accumulation of an isoform of ΔFosB that may be responsible for late response to cellular damage, e.g. delayed apoptosis.

In correlation with the behavioral symptoms of the daily 4-AP seizures described previously, it is plausible that the strong labeling of FosB in the dentate gyrus in our experiments may also be related to a preconditioning effect of brief, repeated seizures that may prevent the excitotoxic damage. According to our experiments, the 12 days of 4-AP seizures did not induce cell death or gross morphological changes in the hippocampus. However, the initiation of further kindling effect after more prolonged period of repeated seizures could induce epileptogenesis with the consequent damage of the vulnerable regions of the hippocampal formation which coincide with weak FosB expression sites.

5. Alterations in the parvalbumin containing neuronal population

PV expressing neurons are fast-spiking GABAergic interneurons that mediate mainly perisomatic inhibition, and the alteration of their function is linked with either hippocampal

and neocortical seizure activity (Silva et al., 2002; Sloviter, 1991). Long-lasting seizures cause neuronal damage that has been reported to occur even in the dentate gyrus among parvalbumin-positive interneurons (Kobayashi and Buckmaster, 2003).

The parvalbumin containing neuronal population has been examined earlier (Mihály et al., 1997, 2001). According to issues related to the acute 4-AP model, the seizure activity induced a long-lasting c-fos expression in parvalbumin-containing hilar neurons in comparison with the granule cells (Mihály et al., 2001). These results were attributed to a possible long-lasting alteration or future cell death, which would be plausible considering that PV-positive cells in the rat hippocampus express protein subunits of the delayed rectifier K^+ channel, and the neurons are sensitive to low concentrations of 4-AP (Du et al., 1996).

Moreover, long-lasting seizures have been shown to induce heat-shock protein expression in hilar neurons, as an indication of cellular injury (Sloviter and Lowenstein, 1992). However, no change in the PV mRNA content was detected in the hippocampus and neocortex after 12 days of 4-AP treatment (Vizi et al., 2004), and the present study revealed no significant reduction in the number of PV immunopositive neurons in the same conditions. The increased immunopositivity of FosB in this neuronal subpopulation could be regarded as indicating the persistent, and probably adaptive, changes in the function of the inhibitory circuits after repeated 4-AP seizures.

According to our results, the hippocampal double labeled cells were increased in number in the CA1 and hilum. Similarly, in the SE model induced by electrical stimulation, GABA-mediated inhibition is often diminished in the dentate gyrus or CA1 area despite the apparent survival of GABA-containing interneurons (Morimoto et al., 2004). Doherty and Dingledine (2001) have provided evidence that basket cells might become dormant due to a weakening of the excitatory drive to hilar interneurons in response to kainate-induced SE. This effect appeared to be mediated by use-dependent alterations in presynaptic glutamate receptors. This is a possible explanation also for the chronic 4-AP seizure model, as the increased FosB expression in PV-positive cells may alter their excitability by rearrangement of glutamate receptor subunits.

6. Changes of PV expression in the MM after repeated 4-AP seizures

Western blotting analysis indicated a significantly decreased amount of PV in MM at 12 days. Remarkably, the degrees of PV mRNA loss detected by in situ hybridization and the decrease in PV protein content in group MM 12 days were very similar: 77% and 79.9%, respectively

(Vizi et al, 2004). As no decreased cell number were observed in the mammillary nuclei in the 12 days' groups, these data indicated a coordinated downregulation of PV at the transcriptional and translational levels.

MM, a major element of the Papez-circuit, receives input from the rostral subiculum and harbours neurons which fire in phase with hippocampal (especially with CA1) theta rhythm (Kocsis et al, 1994). Due to these properties, MM is sensitive to hippocampal seizures and likely to be involved in propagating epileptic activity through the mammillothalamic pathway (Mirski and Ferrendelli, 1987). In fact, it has been shown experimentally that mammillary nuclei and their closely associated structures are selectively labeled with ^{14}C-deoxyglucose in animals pretreated with ethosuximide and challenged with the convulsant agent pentylenetetrazol (Mirski and Ferrendelli, 1986). Further, lesion of the mammillothalamic tract was highly protective against pentylenetetrazol-induced seizures (Mirski and Ferrendelli, 1987). These data indicated that hippocampal epileptic seizures can selectively activated mammillary neurons.

The convulsive agent 4-AP is known to induce glutamate and GABA release in the hippocampus accompanied by epileptiform EEG waves (Fragozo-Veloz et al., 1990). 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 (Allen and Hopkins, 1989). 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 (Scotti et al, 1997). 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 (Buckmaster and Dudek, 1997). However, kainate is known to induce not only seizure, but also excitotoxic cell death in the hippocampus (Buckmaster and Dudek, 1997).

The role of PV in neuroprotection is not clear: some experimental data support the protective role, others do not (D'Orlando et al., 2002). 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 Ca^{++} buffer modulates short-term synaptic plasticity, by enhancing depression and decreasing facilitation (Caillard at al., 2000).

The results obtained on Purkinje cells of PV knockout mice indicated that the absence of PV might cause cumulative facilitation, because Ca^{++} fluxes are not regulated properly (Caillard et al., 2000).

The MM contains projection neurons—the GABAergic innervation of the rat MM originates from midbrain sources and from the surrounding hypothalamic areas (Allen and Hopkins, 1988). The high PV content of the MM neurons can be attributed to the numerous Ca^{++} conductances, which are operative in the bursting activity of these cells (Alonso and Llinas, 1992). Possibly, the PV content buffers the intracellular Ca^{++}, regulates facilitation and depression (Caillard et al, 2000) and protects against the damaging aftermath of Ca^{++} 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 Ca^{++} effects, which later, may lead to the damage and death of these cells. 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 (Corsellis and Meldrum, 1976).

7. Conclusions

- The acute 4-AP seizure model provided a tool for the study of the development of generalized seizure activity in the rat brain. Time-dependent expression of neocortical c-fos correlated with electrophysiological analysis supported the importance of the thalamo-corticothalamic circuit in the activation and maintanace of the neocortical seizure, and suggested a proconvulsant role of the transcriptional activity of c-fos. The ablation of the entorhinal cortex is followed by the suppression of seizure activity and c-fos expression, suggesting the importance of the limbic structures for seizure generation after systemically applied K^+ channel blocker.
- Daily injections of the convusing dose of 4-AP demonstrate changes in excitability and long-lasting expression of FosB. The symptoms of the first 4-AP treatment were usually characterized by the development of generalized tonic-clonic seizures. Then, after an interval of tolerance to seizure occurence of sensitization was observed followed by a further desensitization period towards the 12th day. This is in accordance with literature data concerning the differential regulation of gene

expression by FosB and its isoforms depending on the duration and level of expression. Therefore, the expression of FosB appeared to have protective effect against seizure and cell damage in an initial period of repeated brief generalized seizure sequence, and the cortical expression involved mainly the neocortex and dentate gyrus and proved to be protective also for the hippocampus proper where no cell death was observed.

- A large number of parvalbumin containing cells expressed FosB in the neocortex and regions of the hippocampal formation, suggesting plasticity changes of inhibitory neurons after chronic seizure activity. In our experiments, the only brain region presenting alteration of PV expression was the medial mammillary nucleus, where the preserved number of PV positive cells had significantly decreased PV content.

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SUMMARY

The present study deals with certain aspects involved in the mechanism and consequences of seizure actvity. Transcriptional regulation of gene expression is known to initiate plasticity changes in response to neuronal stimulation, determining different behavioural consequences depending on the temporal pattern of the stimuli. Therefore, we attempted to investigate the significance of expression of transcription factors in relation with behavioural and cellular alterations in response to seizures, in acute and chronic conditions.

The convulsant used in our experiments was the K^+ channel blocker 4-AP, and it was injected intraperitoneally to adult rats, causing generalized tonic-clonic convusion. Immunohistochemical analysis of the c-fos in acute seizure model revealed a certain temporal pattern in the neocortex, which was correlated with mRNA analysis and electrophysiological data. The results indicated a proconvulsive effect attributable to c-fos. The ablation of the entorhinal cortex was followed by the suppression of 4-AP induced seizure activity and attenuated hippocampal c-fos expression, revealing the importance of limbic connections for the development of hippompal seizure.

Daily induced seizures presented a different response at the level of gene expression. Long-lasting expression of FosB transcription factor was detected in the neocortex and dentate gyrus, and the behavioural symptoms suggested changes in the sensitivity for seizure that can be the consequences of altered expression of glutamate receptor subunits regulated by FosB gene products. An overall protective role of FosB was suggested by our results of the chronic seizure experiments. However, the daily seizures caused decrease in the parvalbumin content in the medial mammillary nucleus after the 12th day of seizure, which raises the possibility of a specific vulnerability of interneurons in this region.

ÖSSZEFOGLALÁS

A jelen munka konvulziós tevékenységre jellemző génexpresszió jelentőségének vizsgálatára irányul. Különböző transzkripciós faktorok növekedett receptormediált kifejeződése meghatározza az ingerlésre adott sejtválaszt, amely az akut, illetve krónikus görcsmodellre jellemző eltérő következményeket is alátámasztja.

A kísérleti állatokat kálium csatorna gátló szerrel, 4-aminopiridinnel kezeltük intraperitoneális injektálással. Akut kísérleti modell vizsgálatánál az azonnali és rövidtávú morfológiai változások markereként ismert c-fos transzkripciós faktor kifejeződését regisztráltuk patkány agykéregben. Immunhisztokémiai analízis segítségével a neokortikális c-fos expresszió dinamikáját vizsgáltuk. Az eredmények elektrofiziológiai adatokkal való összegzése a c-fos prokonvulzív hatására utal.

Neuronális aktivációt jelző faktorként a c-fos expresszió konvulziós tevékenység terjedésének meghatározására is alkalmas. Entorhinális kéreg eltávolítása után a 4-aminopiridinnel előidézett konvulziók elektrofiziológiai és génexpressziós válasza egyaránt jelentősen csökkent a hippocampusban. A rohamok enyhülése a c-fos expresszió hippokampális csökkenésével párhuzamos volt, ami a hippokampuszban keletkező epileptogén folyamatokban résztvevő limbikus összeköttetések fontos szerepét jelzi.

Ismételt rövid konvulziók által előidézett tartós és késői génexpressziós változások markereként FosB transzkripciós faktor cellulláris lokalizációját vizsgáltuk patkány agykéregben. Immunhisztokémiai módszer alkalmazásával különbségek mutatkoztak allokortikális és neokortikális struktúrák között. Irodalmi adatok alapján az emelkedett FosB expresszió korrelál a glutamát receptor alegységek átszervezett disztribúciójával, ami az ismételt ingerlésre adott csökkenő neuronális választ eredményezheti.

A gátló neuronális aktivitás szerepét parvalbumin tartalmú interneuronok kimutatása segítségével analizáltuk. FosB és parvalbumin kettős immunjelölés során különböző kortikális területeken tapasztaltunk a kontroll adatoktól való eltérést.

Naponta előidézett konvolziók esetében parvalbumin mRNS és fehérje kimutatását terveztük in situ hibridizáció, Western blot és immunhisztokémia alkalmazásával, melyek során szignifikáns változást tapasztaltunk a corpus mammillare mediale területén.

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