

Neocortical *c-fos* mRNA transcription in repeated, brief, acute seizures: Is *c-fos* a coincidence detector?

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Abstract. The effect of acute brief seizures on neocortical *c-fos* expression was investigated in rats injected with 5 mg/kg 4-aminopyridine. Electroencephalography in freely moving animals with implanted neocortical electrodes detected an average of 2.67 tonic-clonic convulsions within 1 h following the 4-AP treatment. Tissue samples of the somatosensory neocortex were collected at 30 min, 1 h, 3 h, 5 h and 8 h following the treatment for PCR and immunohistochemistry. The *c-fos* mRNA displayed the first significant rise at 1 h, and remained significantly higher through 3 h. The number of *c-fos* protein immunoreactive cells was significantly elevated already at 30 min, peaked at 1 h, and declined by 5 h. We conclude that in repetitive, brief seizures, the first convulsion does not increase *c-fos* RNA transcription, whilst the second causes a long-lasting gene expression and a large increase of *c-fos* protein synthesis. The phenomenon may have implications in the pathogenesis of human and animal epilepsies.

Introduction

In human epileptic case histories, the first symptom towards a long-lasting pathological change is very often an acute, unprecedented convulsion. Patients experiencing 1-2 unprecedented, idiopathic seizures have an increased risk for the development of epilepsy (1). Therefore, the detailed analysis of the molecular events of an acute seizure might provide useful information concerning the pathogenesis of epilepsy. The expression of the Fos family member proto-oncogenes is induced in every animal seizures studied so far, indicating that the activation of the *c-fos* promoter may be an

early common pathway in the convulsive state (2-5). Therefore, combined with the electrophysiological analysis of the acute seizure on freely moving animals, we applied the reverse transcription polymerase chain reaction (RT-PCR) to detect time-related changes of the transcription of the *c-fos* gene, and used immunohistochemistry to track the translation of *c-fos* protein. We compared the transcription dynamics of the *c-fos* gene to the appearance of *c-fos* immunoreactive (*c-fos*IR) cell nuclei, and to the electrophysiological and behavioural symptoms. In the present experiments, we utilized 4-aminopyridine (4-AP), a specific K⁺-channel blocker, which precipitates well-described acute behavioural convulsions in experimental animals (5-7).

Materials and methods

Male Wistar rats (200-250 g bwt) bred in the Central Animal House of Szeged University were used. The animals were kept in standard conditions with free access to water and food. The animal experiments were in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC) and the Hungarian Animal Act (1998). Seizures were induced with a single intraperitoneal (i.p.) injection of 4-AP (Sigma; 5 mg/kg 4-AP, dissolved in physiological saline, 1.0 mg/ml concentration). Control animals received the same volume of physiological saline, i.p. (Table I).

The animals were deeply anesthetised with Nembutal (50 mg/kg, i.p.) and their heads fixed in a stereotaxic frame. Stainless steel screw-electrodes were implanted over the somatosensory cortex (trunk and hindlimb regions) on both sides according to the stereotaxic coordinates (8). An additional screw electrode over the cerebellum served as a reference. Electrode leads were attached to a miniature socket fixed to the skull with dental acrylic cement. Following a 10 day-recovery period, the electrodes were connected to the recording instrument through slip-ring assemblies. The behaviour of the animals was monitored continuously with video cameras.

Spontaneous EEG signals were recorded before seizure induction. The EEG activity was recorded continuously for 8 h following the 4-AP treatment. Signals were amplified 1000-fold with a commercial multichannel amplifier, filtered between 0.3-50 Hz, digitised at a sampling rate of 100 Hz

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Table I. Experimental groups and the number of animals.

Experiments	Number of rats in the control (saline-treated) groups	Number of rats treated with 4-AP	Recording/sampling times after 4-AP injection and in controls after saline injection
Electrophysiology (EEG)	(self control)	5	30 min, 1 h, 3 h, 5 h, 8 h
Immunohistochemistry	15	15	30 min, 1 h, 3 h, 5 h, 8 h
Gene expression analysis (RT-PCR)	15	15	30 min, 1 h, 3 h, 5 h, 8 h

Table II. Features of the EEG seizure activity following 4-AP administration.^a

	Duration of first seizure (sec)	Duration of first tonic phase (sec)	Duration of first clonic phase (sec)	Duration of second seizure (sec)	Duration of second tonic phase (sec)	Duration of second clonic phase (sec)	Frequency in tonic phases (Hz)	Frequency in clonic phases (Hz)
Mean (n=5)	53.53	7.38	46.15	73.44	8.96	64.48	7.77	5.23
SD	4.21	4.10	5.12	13.94	5.26	15.30	1.21	0.98

^aNo statistical analysis was performed on the EEG data.

and stored on computer for off-line analyses. Epileptiform discharges were analysed with custom written software.

In the RT-PCR experiments, we used 30 rats: three controls and three 4-AP-treated animals at each sampling time (Table I). The 4-AP treated rats always displayed behavioural seizures. The rats were decapitated under diethyl-ether anaesthesia, the brains were dissected, samples of the somatosensory neocortex were frozen in liquid nitrogen, homogenized, and total RNA was extracted by the AGPC method (9). The reverse transcription (RT) was made from 2 µg RNA (10). The RT product (1 µl of the 20 µl) was submitted to multiplex polymerase chain reaction (PCR) in 50 µl volume of Taq reaction buffer containing 0.05 µM glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, 0.5 µM *c-fos* primers, 200 µM dNTP, 1.5 mM MgCl₂ and one unit of DNA polymerase. The sequence of GAPDH primers (10) and the sequence of *c-fos* primers (11) have already been described. Amplification was carried out in 25 cycles after carefully establishing the linearity for both the GAPDH and *c-fos* fragments between 20 and 30 cycles. Identity of the *c-fos* PCR fragment (256 bp) and the GAPDH fragment (377 bp) was confirmed by cloning into and sequencing a pGEM-T easy vector. The RT-PCR products were separated on 6% acrylamide gel and stained with ethidium bromide. Densitometric scanning using ImageQuant (Typhoon™ 9400 Variable Mode Imager, Molecular Dynamics) was performed for the quantification of the bands. The Student's t-test and Newman-Keuls test were used for statistical analysis. The level of *c-fos* transcripts in the samples was normalized to the level of GAPDH mRNA detected from the same amplification.

For immunohistochemistry, brains were fixed by transcardiac perfusion (4% paraformaldehyde in 0.1 M phosphate buffer; pH 7.4). 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 anti-rabbit IgG (1:40; Jackson Immuno-Research, PA, USA). The secondary antibody was detected by PAP complex diluted to 1:1000 (Jackson). The peroxidase reaction was localised with nickel chloride-containing diaminobenzidine tetrahydrochloride (Sigma), yielding a black reaction product.

From every animal, five coronal immunohistochemical sections of identical stereotaxic coordinates were selected. Within the areas of interest (AOIs), the immunoreactive cell nuclei were counted using a Nikon Eclipse 600 microscope equipped with a Spot RT Slider digital camera (1600x1200 dpi in 8 bits), using the Image Pro Plus 4.5 morphometry software (Media Cybernetics, Silver Spring, MD, USA). The counting was performed blinded to the animal treatment. Cell counts in the S1Tr region of the neocortex (8) were done by using a 10x objective. The AOI size (0.25 mm²) was the rectangular image-capturing field of the camera which included each neocortical layer (I-VI), so that the layers could be evaluated separately. Frozen sections stained with cresyl violet were used as reference for the thickness of the neocortical layers. The data were analysed statistically comparing sets of findings obtained with the same magnification. Cell counts were normalised to 1 mm². Differences in the number of c-fosIR cells in the control and convulsing animals were analysed with one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. A significance criterion of 0.05 was used. The statistical analysis was performed with SPSS 9.0 software.

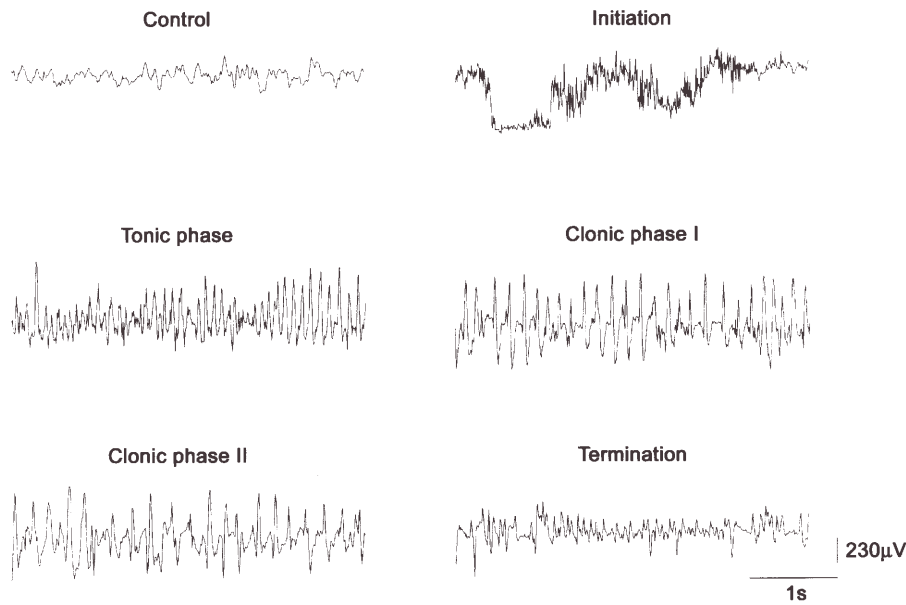


Figure 1. Following 4-AP treatment, neocortical seizure activity appeared in each animal. The representative parts of the records are from control animals, before the injection of 4-AP (Control), and during the seizure (Initiation, Tonic phase, Clonic phase I, Clonic phase II, Termination). Time and voltage scales are shown.

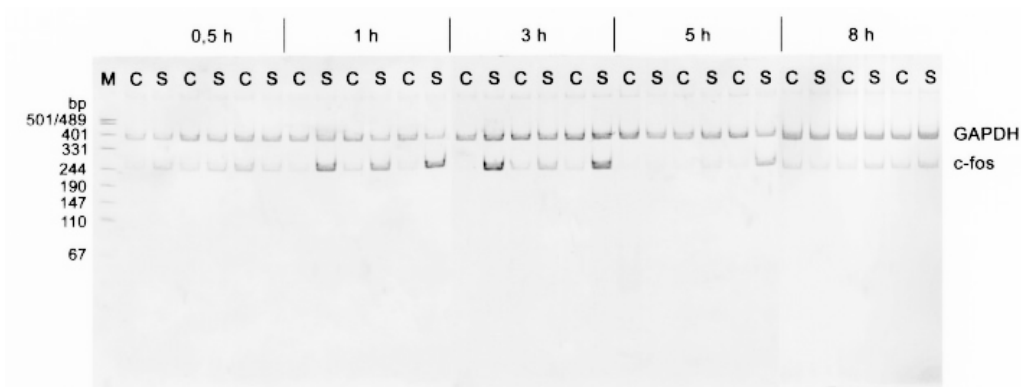


Figure 2. Polyacrylamide gel analysis of fragments of multiplex RT-PCR reactions. The upper bands show GAPDH, the lower bands *c-fos*. The first lane (M) displays length markers. S, neocortex of 4-AP-treated animals; C, neocortex of rats injected with physiological saline.

Results

The i.p. administration of 4-AP induced generalised tonic-clonic seizures. During the 8-hour-recording period, two seizures were detected in each animal except one, in which three events occurred. The convulsions appeared during the first hour of the observation. The behavioural sequel of 4-AP seizures was described in detail previously (7). The first seizure activity appeared on the EEG with an average latency of 20.70 ± 6.48 min. This event was followed by a second seizure, and the mean delay between the two seizure events was 16.22 ± 4.86 min. The mean duration of the first discharges was 53.53 ± 4.21 sec, while the second was significantly longer, lasting for 73.44 ± 13.94 sec (Table II). Repetitive spikes characterized each seizure event in the so-called tonic phase and repetitive spikes at a smaller frequency, as well as spike-and-wave complexes in the so-called clonic phase

(Fig. 1, Table II). The EEG seizure and the behavioural symptoms did not last longer than 1 h.

The mRNA of *c-fos* was detectable in the neocortex of control and 4-AP treated rats, as was the internal control GAPDH mRNA (Fig. 2). In control and 4-AP-treated rats, GAPDH messages were not different. At 30 min following the treatment, no increase of the intensity of the *c-fos* mRNA signal was detected. At 1 h following the 4-AP treatment, the level of *c-fos* mRNA increased significantly ($P=0.0134$). This increase of *c-fos* mRNA in 4-AP treated animals was also maximal and highly significant, compared to levels detected at other time points (Fig. 3). At 3 h, the increase (compared to controls) was at the border of significance ($P=0.0617$). After that, *c-fos* mRNA decreased gradually below the control level by 8 h (Fig. 3).

The c-fosIR cell nuclei were present in every layer of the neocortex following 4-AP administration (Fig. 4). The increase

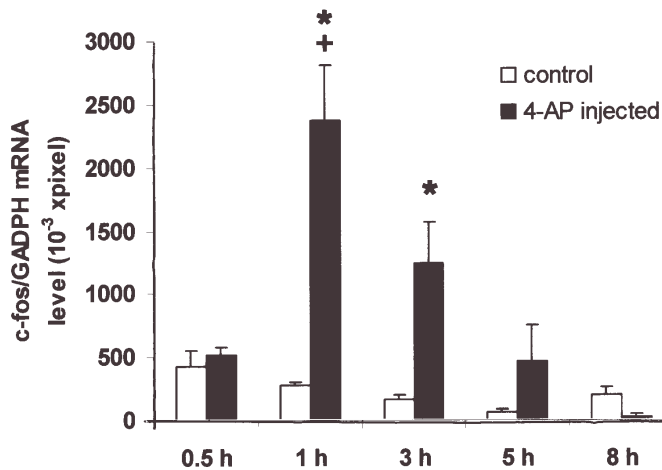


Figure 3. Diagram of *c-fos* mRNA levels after normalization to the coamplified GAPDH mRNA. Black columns show the mean values of 4-AP treated animals, white columns show the mean values of physiological saline treated rats, vertical bars represent \pm SE. The differences compared to controls are significant at 1 and 3 h (*). The differences between convulsing animals are very significant at 1 h compared to 0.5, 5 and 8 h (+). Labels: (+) very significant difference (Newman-Keuls test, $P=0.0134$); (*) significant difference (paired t-test, $P=0.0617$).

of the number of *c-fos*IR 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 (Fig. 5). The highest elevation in the number of *c-fos*IR cells at 30 min was seen in layer IV: other layers contained less *c-fos*IR 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 slightly in layers II, III, IV, V and VI. At 5 h, the cell numbers decreased to control levels, and remained so at 8 h (Fig. 5). The values in convulsing animals displayed highly significant differences ($P \leq 0.01$) compared to the controls (Fig. 5). In controls, scattered *c-fos*IR cells were seen in layer IV (Fig. 5).

Discussion

Previous studies from our laboratory indicated that the 4-AP model is reliable for the investigation of seizure genesis, and that careful counting of *c-fos*IR cell nuclei serves as an indicator of seizure spread in forebrain structures, *in vivo* (5,12). In the present experiments, the RT-PCR studies demonstrated the time-related induction, increase and decrease of the *c-fos* mRNA in the cerebral cortex following 4-AP injection. This is the first such report in the literature of 4-AP seizures. Another important observation in the present experiments is that the activation of the *c-fos* gene lasted significantly longer than the electrophysiological and behavioural seizure.

In our experiments, one dose of intraperitoneally applied 4-AP induced at least two seizure events in the animals: this is what we call brief, repetitive convulsions. In a similar series of experiments with 5 mg/kg 4-AP, only 70% of the rats was affected and usually only one tonic-clonic seizure

was detected (6). Compared to our results, the latency of the seizures was similar, only the duration of the events was somewhat shorter (6).

Timing of the events was relatively uniform; SD was only 6.48 min in case of the first seizure and 4.86 min for the time between the seizures. These values are similar to that we detected with the measuring of the latencies of the symptoms: in those experiments, the latency of the first tonic-clonic seizure was 25.8 ± 1.9 min (7). The standard latency of the first generalised convulsion proves the reliability of the 4-AP model: indicates that 4-AP penetrates the brain barriers evenly, and that the elimination of the drug follows a standard kinetics (13). Our findings concerning the appearance of *c-fos* in the convulsing brain are in accordance with previous reports (2,4).

The duration of the second EEG seizure was longer than that of the first significantly, indicating the sensitization of large neuronal populations following the first convulsion. In the neocortex, pyramidal cell interactions are very effective, probably because of the high cellular density and high degree of axonal collateralization (14). The pyramidal-to-pyramidal cell excitatory synapses mainly use NMDA receptors; therefore they exhibit self-facilitation (14). The result is the reverberant excitatory activity within a pool of interconnected pyramidal cells (14). Our recent experiments with *in vivo* microdialysis proved that 4-AP convulsions were accompanied by a large and long-lasting increase of glutamate release (15). Moreover, seizure events and *c-fos* expression were dependent on NMDA-type glutamate receptors (5). Therefore, we think that the sharp increase of *c-fos* mRNA in the neocortex reflects the activation of ionotropic (mainly NMDA-type) glutamate receptors.

However, the effects of the two repetitive seizure episodes were different. The first seizure (appearing at 20 min) did not generate an increase in *c-fos* mRNA level, as measured at the 30 min RT-PCR sampling time. The second seizure (appearing 30-40 min after the injection) boosted the transcription of *c-fos*, causing a highly significant elevation at 1 h, as shown by the RT-PCR measurements.

On the other hand, 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 (16). 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 (16) 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 (17). This layer retained a relatively high number of stained cells after 3 h, suggesting that the thalamocortical-corticothalamic circuits are important in the maintenance of synchronized neocortical activity (18). Our previous rCBF measurements proved the large increase of rCBF in the dorsal diencephalon and the neocortex in 4-AP seizures in mice (19). Following this line of evidence, we conclude that in acute

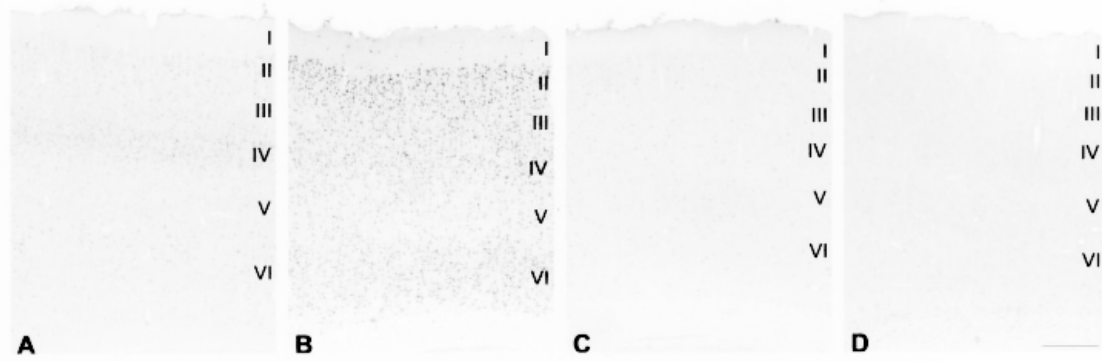


Figure 4. Immunohistochemistry of *c-fos* protein in the S1Tr neocortex in 4-AP-injected animals (A, 30 min; B, 1 h; C, 3 h; D, 5 h). Controls are not shown because they display a staining pattern similar to D. Roman numerals indicate neocortical layers; bar, 500 μm .

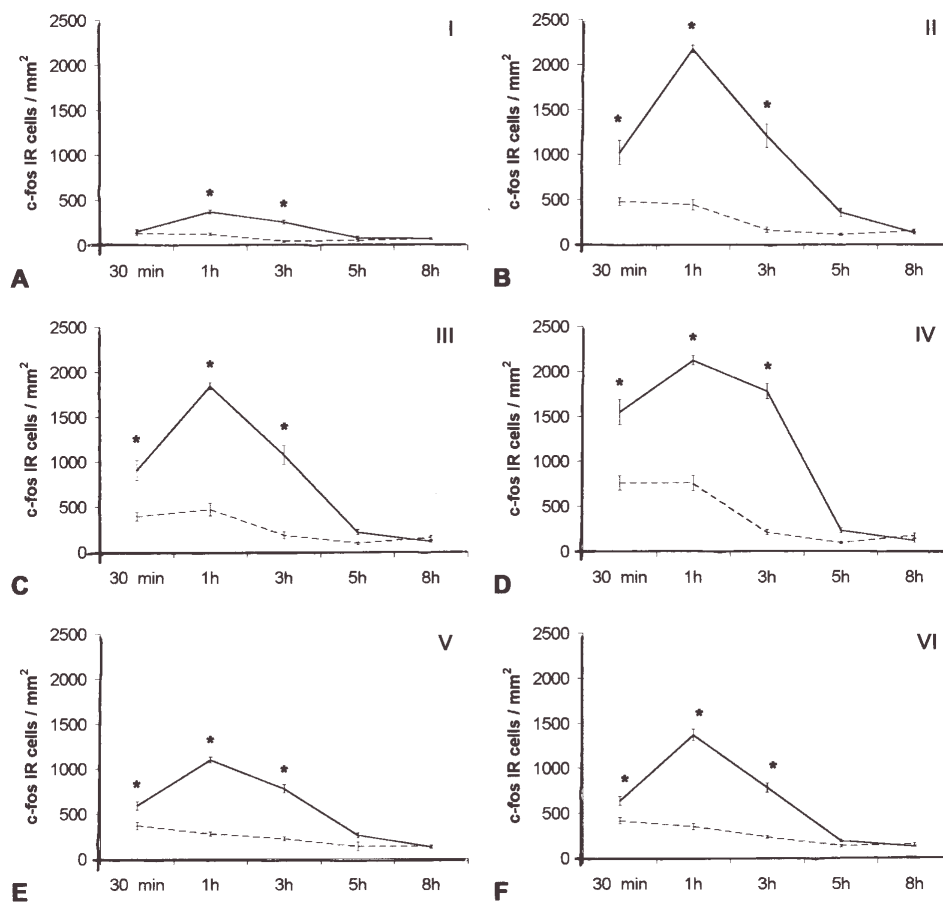


Figure 5. Results of the counting of *c-fos*IR cell nuclei in the neocortex: cell counts in 4-AP seizures at 30 min, 1 h, 3 h, 5 h and 8 h. Solid lines represent cell counts in 4-AP-treated rats, broken lines represent controls and vertical bars represent \pm SE. We noted that the control and 4-AP treated groups differ significantly in every cases at 1 and 3 h (A-F), and also at 30 min in layers II, III, IV, V, VI (B-F). Asterisks denote significant difference ($P < 0.01$). A, layer I; B, layer II; C, layer III; D, layer IV; E, layer V; F, layer VI. Slight, non-specific elevation in control animals was due to the handling during the experiment.

neocortical seizure the first cellular event [mediated by the synchronised activity of thalamocortical afferents (18) during the first EEG convulsion] 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 (3). 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 1 h 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 (14). 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. Literature data prove that the breakdown occurs via the ubiquitin/proteasome pathway, indicating that not only

the transcription and translation but also the degradation are strictly controlled processes (20).

Summarizing our results, we conclude that upon the effect of the first brief convulsion, Fos-proteins move into the cell nucleus, where they bind to the AP-1. Therefore, the next convulsion will find functioning *c-fos*-activated AP-1, and the effects in terms of transcriptional regulation will be greater. Based on these results, we think that *c-fos* (and probably other IEGs) may act as metaconvulsive agents (21), facilitating the forthcoming seizure. We propose that similarly to memory (21), IEGs also act as coincidence detectors, potentiating the long-time cellular effects of successive, brief seizures. This process of potentiation and the coincidence detector effect of the IEGs may have significance in the pathogenesis of those idiopathic human epilepsies, which develop due to unprecedented acute convulsions.

Acknowledgements

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