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Alterations of seizure-induced c-*fos* immunolabelling and gene expression in the rat cerebral cortex following dexamethasone treatment

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Summary

We examined the effects of dexamethasone on the expression of the inducible transcription factor c-*fos* in 4-aminopyridine (4-AP) seizures. Induction of c-*fos* mRNA due to 4-AP-elicited convulsion was detected by means of the polymerase chain reaction (PCR) in samples from the neocortex. Adult male rats were pretreated with different doses of dexamethasone (0.5, 1, 3, 5 mg/kg body weight); 1 h later 5 mg/kg 4-AP was injected intraperitoneally. Controls received the solvent of dexamethasone. Pretreatment with dexamethasone provided significant symptomatic protection against 4-AP-induced convulsions. Immunohistochemistry was used to evaluate the presence of the c-*fos* protein. The number of Fos-immunoreactive nuclei per section area was measured in the neocortex and hippocampus. Pretreatment with dexamethasone resulted in a dose-dependent, significant decrease of seizure-induced Fos-protein immunoreactivity in the neocortex, in the hilum of the dentate fascia, as well as in regions CA1-3 of the hippocampus, compared to control animals. Brains processed for mRNA isolation and PCR, displayed a significant increase of c-*fos* mRNA following the 4-AP treatment, while pretreatment with dexamethasone did not prevent or decrease this boosted c-*fos* mRNA expression. We conclude that seizure-induced c-*fos* expression and intracellular Fos-protein localization are mediated by transmitter and receptor systems, and dexamethasone significantly decreases Fos immunoreactivity, probably by regulating

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the intracellular traffic of the protein. We also conclude that dexamethasone does not interfere with the genomic regulation of c-*fos* mRNA synthesis.

Introduction

Induction of neuronal gene expression has been reported in conditions that lead to neuronal plasticity and permanent changes in brain function, such as learning and memory (Goelet et al., 1985; Greenberg et al., 1986; Morgan and Curran, 1985). In particular, the proto-oncogene c-*fos* can be rapidly induced in response to a variety of stimuli in neuronal cells (Hanley, 1988). Although many of the stimuli act through the regulation of intracellular calcium levels, c-*fos* induction may be a point of convergence for a wide range of conditions. This suggests that different cytoplasmic pathways must interact with regulatory factors that impinge on the c-*fos* gene (Hanley, 1988). The expression of the proto-oncogene c-*fos* is induced in chemically and electrically elicited seizures, indicating that the activation of the c-*fos* promoter may be an early common pathway of the pathological stimuli (Gass et al., 1992; Herdegen and Leah, 1998; Mihály et al., 2005). Expression of c-*fos* in epileptic seizures, is mediated principally by transmitters acting on ionotropic receptors and voltage-dependent calcium channels (Greenberg and Ziff, 2001; Szakács et al., 2003). NMDA (*N*-methyl-D-aspartic acid) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) are the main candidates for the induction of the c-*fos* proto-oncogene (Greenberg and Ziff, 2001). Accordingly, blockade of the NMDA receptor channel decreases seizure-induced c-*fos* expression, which indicates the importance of the postsynaptic effects of glutamate and the concomitant of Ca^{2+} influx (Szakács et al., 2003).

There are extensive data regarding the genomic effects of corticosteroids in the hippocampus. The stress-induced increase in corticosterone secretion leads to structural and functional neuronal changes. Exposure to a high dose of exogenous corticosterone has been shown to cause neuronal atrophy (Woolley et al., 1990). In addition to their classical genomic effects on neuronal structure (effected in part via intracellular steroid receptors), glucocorticoids act acutely on neuronal excitability: they reversibly and biphasically modulate the excitability of hippocampal neurons, and influence the magnitude of long-term potentiation (LTP) (Pavlides et al., 1995; Kerr et al., 1994; Diamond et al., 1992). High concentrations of glucocorticoids suppress the LTP of the population spike within 1 h (Vidal et al., 1986). Additionally, application of corticosterone for 20 min significantly inhibits the development of LTP in the CA1 region of rat hippocampal slices (Kawato et al., 2001; Shibuya et al., 2003). The induction of LTP is related to Ca^{2+} influx via NMDA receptors. It has also been demonstrated that 30 min preincubation of hippocampal slices with corticosterone significantly decreases NMDA-receptor-mediated Ca^{2+} accumulation in CA1 levels (Sato et al., 2004). Data reported in the literature also indicate that corticosteroids have beneficial effects in some animal and human epilepsies (Edwards et al., 2002).

Previous studies from our laboratory indicated that immunohistochemical detection of Fos protein in 4-aminopyridine (4-AP) convulsions serves as an indicator of neuronal hyperactivity and seizure spread in forebrain structures (Mihály et al., 2001, 2005; Szakács et al., 2003). Seizure events and c-*fos* expression were dependent on NMDA-type glutamate receptors (Szakács et al., 2003). Moreover, we detected a slight discrepancy between the increase of Fos-immunolabeled cells and the increase in c-*fos* mRNA level, indicating, that the seizure signals act not only at genomic, but also at cytoplasmic levels (Mihály et al., 2005). In the experiments reported here, we investigated the possible beneficial effects of dexamethasone, a highly potent synthetic corticosteroid, in seizures elicited by 4-AP. We measured the expression of the c-*fos* gene, using reverse transcription (RT) polymerase chain reaction (RT-PCR) to detect c-*fos* mRNA. At the same time, we investigated the intracellular appearance and distribution of the Fos protein by means of semi-quantitative immunohistochemistry.

Material and methods

Animal treatment

Male Wistar rats weighing 220–280 g, bred in the Central Animal House of Szeged University were used. The animals were housed under standard conditions with free access to water and food. The

Table 1. Experimental groups and the number of animals

Experiments	Number of rats treated with the solvent of 4-AP (0.9% NaCl)	Number of rats treated with 4-AP	Number of rats treated with 4-AP+solvent of dexamethasone	Number of dexamethasone-pretreated plus 4-AP injected rats				Rats treated only with dexamethasone	Rats treated only with the solvent of dexamethasone
				0.5 mg/kg	1 mg/kg	3 mg/kg	5 mg/kg		
Behavioural analysis	—	—	10	10	10	10	10	—	—
Immunohisto-chemistry	—	—	10	10	10	10	10	12	10
RT-PCR	3	3	—	—	—	—	3	—	—

experiments were conducted in accordance with the Hungarian Animal Act (1998). Written permission was obtained from the Faculty Ethical Committee on Animal Experiments, University of Szeged.

For immunohistochemistry, a total of 81 animals were used. To study the potential dose-dependent effects of dexamethasone, experiments were performed on animals in four groups, each of ten animals. Dexamethasone (Sigma, USA) was dissolved in 30% ethanol and injected intraperitoneally (i.p.) at doses of 0.5, 1, 3 and 5 mg/kg body weight (b.w.), respectively (40 animals). One hour after the administration of dexamethasone, seizures were induced with a single i.p. injection of 4-AP (Sigma), 5 mg/kg 4-AP, dissolved in physiological saline at a concentration of 0.67 mg/ml. In our previous investigations, this dose proved to be epileptogenic (Mihály et al., 1990; Szakács et al., 2003). Another experimental group (10 animals) received similar volumes of 30% ethanol (the solvent in which dexamethasone was dissolved in for experimental animals), then, 1 h later, 5 mg/kg b.w. 4-AP. The control group (consisting of four sub-groups of three animals each, giving a total of 12 animals) received only dexamethasone (0.5, 1, 3 and 5 mg/kg b.w.) dissolved in 30% ethanol. Finally, an additional control group of 10 animals received only the solvent, 30% ethanol. Details of all groups are summarized in Table 1. The behavioural outcome of the experiments, and in particular the latency of the onset of generalized tonic-clonic seizures (GTCS) were recorded from the time of 4-AP injections.

At the end of the experiment (the control animals were sacrificed 1 h after the dexamethasone and/or ethanol injections), 1 h after the i.p. injections of 4-AP, the animals were deeply anaesthetized with diethyl-ether and perfused transcardially with 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 4% phosphate-buffered paraformaldehyde, pH 7.4, as a fixative. The brains were removed and postfixed in 4% paraformaldehyde for 24 h at room temperature. Following postfixation, the brains were cryoprotected overnight in 30% sucrose in PBS, pH 7.4. 24 μm-thick serial sections were cut, between the interaural coronal planes 5.40 and 6.70 mm (Paxinos and Watson, 1998) using a freezing microtome (Reichert-Jung Cryocut 1800), and every fifth section was processed for immunohistochemistry.

C-*fos* mRNA detection

In the RT-PCR experiments, which were performed similarly to the behavioural and immunohistochemical

experiments, we used three rats in each experimental group. Controls received the solvent of 4-AP (0.9% NaCl in distilled water, i.p.), and were sacrificed 1 h after the injection. The next three animals were treated with 4-AP (5 mg/kg, i.p.) and were sacrificed 1 h later. Finally, three rats were pretreated with 5 mg/kg dexamethasone (dissolved in 30% ethanol and administered i.p.), and after 1 h they were injected with 5 mg/kg 4-AP. These animals were sacrificed 1 h following the 4-AP injection (see Table 1). We did not investigate the effects of 30% ethanol in these experiments. We used the 5 mg/kg dexamethasone dose, because the effects of this dose on Fos protein expression were the most pronounced (see Results). The rats were sacrificed by decapitation under deep diethyl-ether anaesthesia. The brains were then dissected on ice, and samples of the neocortex were immediately frozen in liquid nitrogen. After homogenization of the tissue samples, the total RNA was extracted by the AGPC method (Chomczynski and Sacchi, 1987). The RT was made from 2 μg of RNA (Zádor et al., 1996). The RT product (1 μl of the 20 μl) was submitted to multiplex PCR in 50 μl volume of Taq reaction buffer containing 0.0.5 μM glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, 0.5 μM c-*fos* primers, 200 μM dNTP, 1.5 mM $MgCl_2$ and one unit of DNA polymerase. The sequence of GADPH primers (Zádor et al., 1996) and the sequence of c-*fos* primers (Arrieta et al., 2000) have been described. Amplification was carried out in 25 cycles after 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 pGEM-T easy vector and sequencing. The RT-PCR products were separated on 6% acrylamide gel and stained with ethidium bromide. Quantification of the bands was performed by densitometric scanning, using ImageQuant (Typhoon™ 9400 Variable Mode Imager, Molecular Dynamics, GE Life Sciences). The Student's *t*-test and Newman–Keuls multiple-comparison test were used for statistical analysis. The levels of c-*fos* transcripts in each of the samples were normalized to the level of GAPDH mRNA detected from the same amplification reaction.

Immunohistochemistry and analysis of the immunohistochemical data

A streptavidin-peroxidase immunodetection technique was used to localize c-*fos*. Sections were pretreated with 2% H_2O_2 in 0.5% Triton X-100 to block endogenous peroxidase, and then incubated successively in 20% v/v normal pig serum, then primary polyclonal antisera raised against c-*fos* (raised in rabbit; Santa Cruz Biotechnology, USA), diluted 1:2000 in 20% v/v normal pig serum, and then in donkey anti-rabbit IgG (Jackson Immuno-Research, USA), diluted 1:40. The secondary antibody was detected using streptavidin-peroxidase (Jackson Immuno-Research), diluted 1:1000. All dilutions and washes between incubations were performed in PBS (pH 7.4). The enzyme label, peroxidase, was detected using nickel-chloride-containing diaminobenzidine tetrahydrochloride (Ni-DAB kit; Sigma) substrate, applied according to manufacturer's instructions, which yielded a black reaction product. No counterstaining was employed. The sections were dehydrated through a graded ethanol series, cleared in xylene and mounted with Entellan® (Fluka).

Quantitative analysis was performed on five sections from every animal. The structures were identified using stereotaxic coronal plane diagrams (Paxinos and Watson, 1998). Areas of interest (AOIs) for immunoreactive cell counts were selected from the neocortex (S1Tr region), regions CA1, CA2 and CA3 of the hippocampus, the hilum and the granule cell layer of the dentate fascia. The AOIs were determined using the rectangular image-capturing field of the camera. Within the AOIs, the immunolabelled neuronal nuclei were counted using a Nikon Eclipse 600 microscope equipped with a SPOT RT Slider digital camera (1600 × 1200 dpi in 8 bits), using the Image ProPlus 4.0 morphometry software (Media Cybernetics, Silver Spring, USA). Following background subtraction, the threshold was adjusted so that all labelled cell nuclei could be recognized. The counting was performed by an observer blinded to the identity of the sample.

In the neocortex, cell counts were done first using a 10 × objective magnification, and the AOI (an area of 1.20 mm^2) included every neocortical layer (I–VI). Cell counts were normalized to 1 mm^2. Then, the different neocortical layers were analyzed using a 40x objective, the AOI being 0.05 mm^2. In the hippocampus, counting was performed using a 40 × objective. In areas CA1-3 of the Ammon's horn, the AOI (an area of 0.05 mm^2) included the *stratum pyramidale*. The hilum of the dentate fascia was outlined manually, according to Amaral (1978), and used as AOI. Cell counts were normalized to 1 mm^2.

The data were analyzed statistically comparing sets of findings obtained with the same magnification. Differences in the number of Fos-immunoreactive nuclei in the control, 4-AP-treated and dexamethasone-pretreated animals were analyzed with one-way analysis of variance (ANOVA), followed by the Bonferroni *post hoc* test.

Table 2. Behavioural analysis of the effect of dexamethasone pretreatment on 4-AP seizures

Animal treatment	GTCS latency (min)	S.E.M.
4-AP+solvent of dexamethasone	10.60	0.48
4-AP+0.5 mg/kg dexamethasone	18.00*	3.19
4-AP+1 mg/kg dexamethasone	18.66*	1.61
4-AP+3 mg/kg dexamethasone	24.28*	2.29
4-AP+5 mg/kg dexamethasone	15.50*	0.99

The tests were conducted in groups of 10 animals each. Dexamethasone was injected i.p. 1 h prior to the application of 4-AP. The latencies of the onset of GTCS were measured from the time of the 4-AP injection. Significant differences are indicated with asterisks (*).
GTCS, generalized tonic-clonic seizures; S.E.M., standard error of the mean.
*$p<0.05$; ANOVA followed by the post hoc Bonferroni test.

A significance criterion of 0.05 was used. The statistical analysis was performed using the SPSS 9.0 computer programme.

Results

Behavioural analysis

As described in detail previously (Mihály et al., 1990, 2001; Szakács et al., 2003), the i.p. administration of 4-AP caused characteristic behavioural symptoms which culminated in GTCS. The symptoms of the first GTCS were always sudden and clear-cut; therefore, the latency measurements were reliable. Data are summarized in Table 2. The latencies of the GTCS increased in the animals pretreated with dexamethasone: the values changed significantly in all pretreated groups. The most effective in this respect was pretreatment with 3 mg/kg dexamethasone, with an average latency of 24.29 ± 2.3 min (see Table 2). However, the GTCS was not eliminated, and the symptoms preceding GTCS (tremor of the vibrissal and masticatory muscles, generalized tremor) were similar in the 4-AP-treated and dexamethasone-pretreated animal groups. Dexamethasone alone and the 30% ethanol solvent of dexamethasone did not result in any alteration of animal behaviour (the amount of 30% ethanol injected was between 0.6 and 0.8 ml, depending on the body weight of the animal).

Expression of c-*fos* mRNA

The mRNA of c-*fos* was detectable in the neocortex of saline-treated, 4-AP-treated and dexamethasone-pretreated rats, as was the internal control GADPH mRNA, illustrated in Fig. 1. In saline-treated controls, minimal, basal c-*fos* mRNA expression was detected. 1 h following the 4-AP injection, the level of c-*fos* mRNA increased significantly compared to levels in the saline-treated control rats ($p<0.05$). The c-*fos* mRNA level in the dexamethasone-pretreated brains was also significantly higher than that of the saline-treated controls ($p<0.05$). There were no significant differences between the c-*fos* mRNA levels of the 4-AP-treated animals and the animals pretreated with 5 mg/kg dexamethasone, as seen in Fig. 1.

Immunohistochemistry

As described in previous studies (Mihály et al., 2001; Szakács et al., 2003), areas CA1-3 of the hippocampus and the granule cell layer of the dentate fascia displayed strong immunolabelling following 4-AP administration, illustrated in Fig. 2. Most of the labelled nuclei in the Ammon's horn were observed in the pyramidal cell layer, whilst the *strata oriens*, *radiatum* and *lacunosum-moleculare* contained few, scattered Fos-positive cell nuclei. High packing density of Fos-labelled nuclei was seen in the granule cell layer of the dentate fascia, so that counting of the activated granule cells was not possible. The hilum contained strongly labelled, activated cells. Application of different doses of dexamethasone 1 h prior to the convulsant agent resulted in significant decrease of positive Fos-immunolabelled cell nuclei in CA1, CA2 and CA3 regions of the hippocampus, as well as in the hilum of the dentate fascia, seen in Fig. 2, and confirmed by the statistical evaluation. The number of immunolabelled cells in these areas was markedly lower at 0.5 mg/kg dexamethasone-pretreatment, and subsequently gradually decreased in a dose-dependent manner.

Fos-immunoreactive cell nuclei were present in every layer of the neocortex, as well as in the hippocampus following 4-AP administration. In the neocortex, strong immunolabelling was detected in layers II, III, IV, V and VI (data not shown). Pretreatment with 0.5, 1, 3 and 5 mg/kg dexamethasone prior to the administration of the convulsant agent resulted in a dose-dependent decrease of Fos-immunoreactive nuclei compared to the 4-AP-treated animals. Regarding the immunolabelling pattern, dexamethasone caused an overall decrease of Fos-positive cell nuclei, as seen in Fig. 2. Quantitative analysis revealed that the

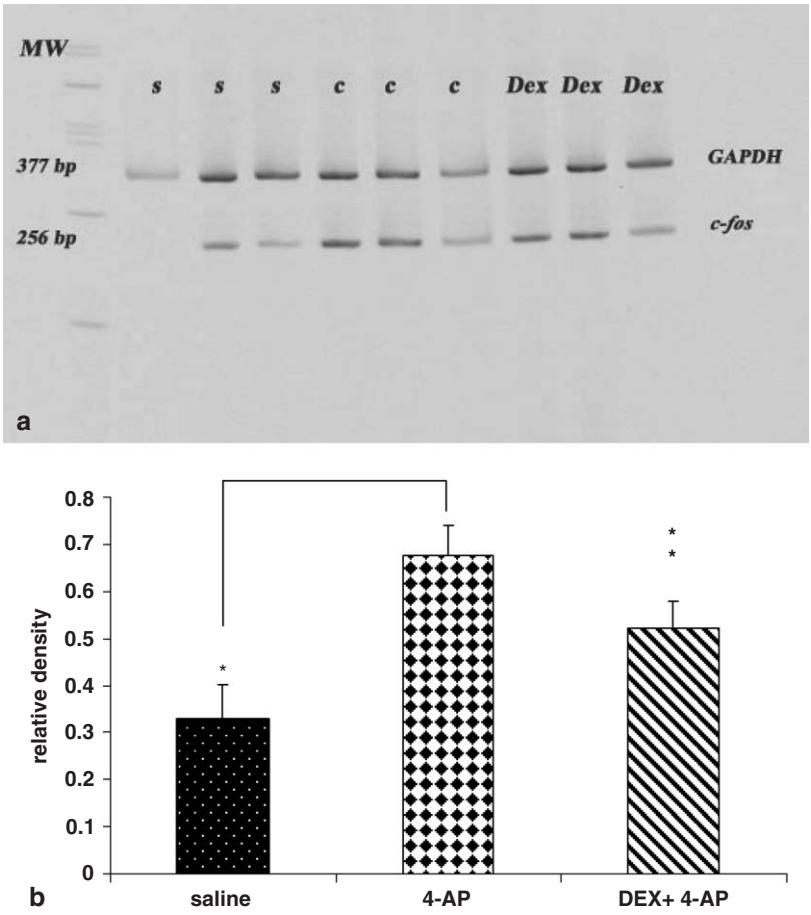

Figure 1. (a) Polyacrylamide gel analysis of c-*fos* mRNA in neocortical samples. The upper bands show GADPH, the lower bands c-*fos*. The first lane (MW) displays length markers; s, saline-treated; c, control (4-AP- treated); DEX, dexamethasone. (b) Histogram illustrating c-*fos* mRNA signal densities in the cerebral neocortex in saline-treated, 4-AP-treated and dexamethasone-pretreated (DEX+4-AP) animals. The columns show the mean values detected by densitometry, the vertical bars indicate the standard error of the mean (S.E.M). The difference compared to saline-treated animals is significant ($^{*}p < 0.05$). Dexamethasone-pretreatment did not cause a significant change in the level of the c-*fos* mRNA in comparison to 4-AP-treated animals. However, the dexamethasone-pretreated group displayed significantly higher values, compared to the saline-control group ($^{**}p < 0.05$).

number of Fos-immunopositive cell nuclei significantly and consistently decreased in dexamethasone-pretreated animals in a dose-dependent manner, and the most pronounced effect was detected at the dose of 5 mg/kg dexamethasone pretreatment (Fig. 2). The analysis of the neocortical layers resulted in similar results; however, there were differences as to the effectiveness of dexamethasone, seen in Fig. 3. Very pronounced inhibitory effects were seen in layers II/III – the largest decrease of Fos immunolabelling was found in layer II. The decrease was very modest (although significant) in layer IV. Layer V reacted similarly: dose dependency was not clear-cut, because the largest decrease was associated with the lowest dose (0.5 m/kg). Layer VI displayed a significant decrease at the lowest (0.5 mg/kg) dose. The effects of the 1 mg and 3 mg doses were similar, but the 5 mg/kg dose resulted in a further decrease in immunolabelling as seen in Fig. 3.

When given alone, dexamethasone did not cause neocortical and hippocampal Fos induction (Fig. 3). The number of Fos-labelled cell nuclei was consistently very low in all the above regions, and the different doses of dexamethasone had no impact on Fos-immunoreactivity (Fig. 3). The effects of ethanol were similar to those of dexamethasone: no change in basal c-*fos* immunoreactivity was detected (data not shown).

Discussion

Our previous studies indicated that immunohistochemical detection and quantitative evaluation of Fos-containing cell nuclei in 4-AP seizures serve

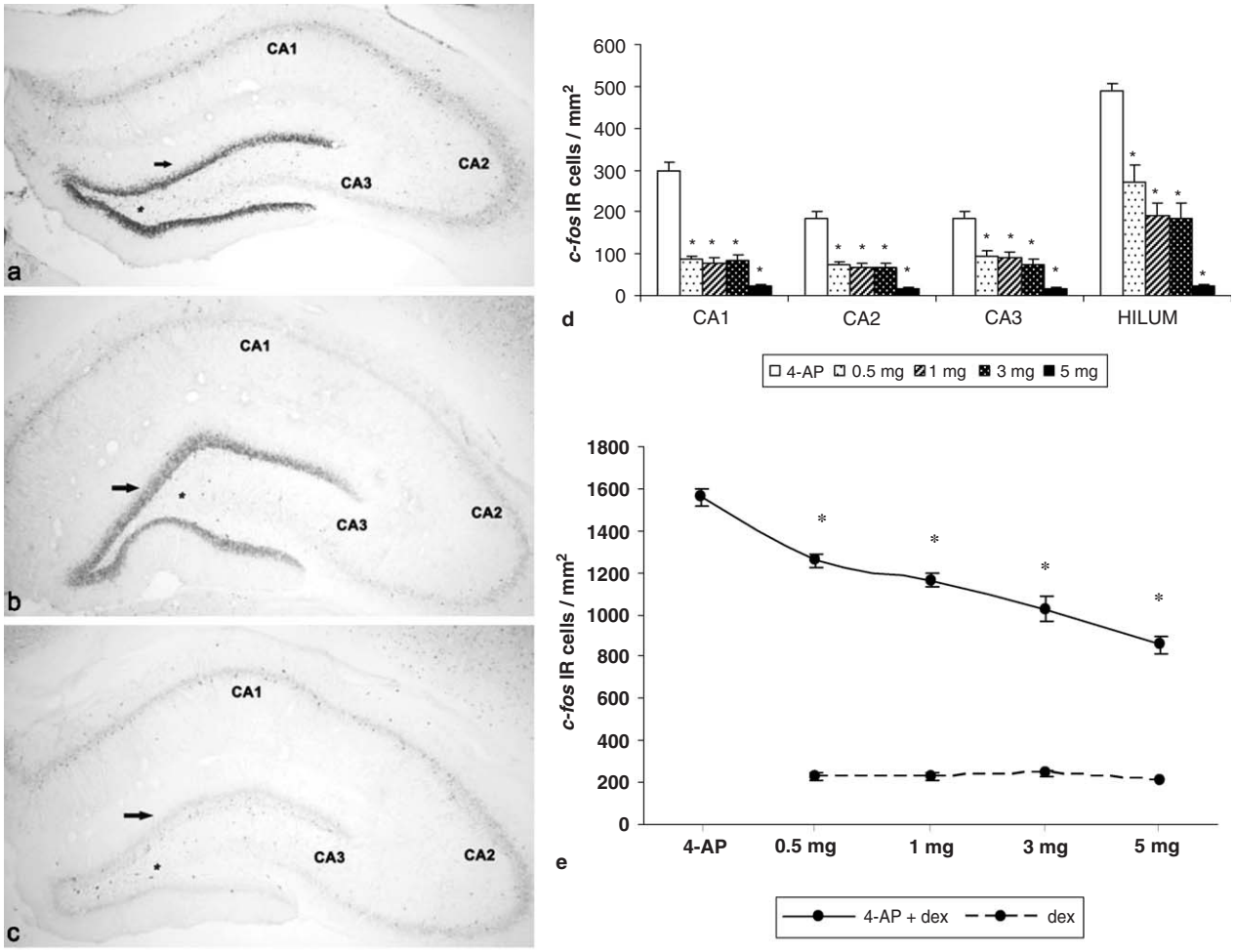

Figure 2. (a–c) Low magnification images of the distribution of Fos-immunopositive cell nuclei in the hippocampus. The sectors of Ammon's horn (CA1, CA2 and CA3) are indicated. Arrow points to the granule cell layer of the dentate fascia, while the asterisk shows the hilum: (a) treated with 4-AP; (b) effect of dexamethasone-pretreatment at a dose of 5 mg/kg; (c) treated only with dexamethasone. (d) Results of cell counts in the hippocampus (CA1, CA2, CA3) and hilum of the dentate fascia in dexamethasone-pretreated and 4-AP-injected rats compared with animals injected with 4-AP only. Asterisks denote significant differences ($p < 0.05$); S.E.M. is indicated in every case; IR, immunoreactive. (e) Results of the counting of Fos-immunopositive cell nuclei in the neocortex in dexamethasone-pretreated animals compared with rats injected with 4-AP. The data were obtained from counting the whole thickness of the neocortex (every layer). The concentrations below the figures indicate the different doses of dexamethasone. Note that the 4-AP-treated and dexamethasone-pretreated groups differ significantly in a dose-dependent manner. Asterisks: significant differences ($p < 0.05$; ANOVA, *post hoc* Bonferroni test); vertical bars: S.E.M. Dotted line indicates the results of control animals, treated with dexamethasone only. There are no significant dose-dependent alterations in these animals.

as an indicator of convulsive activity, and also that the 4-AP model is reliable for pharmacological investigations in vivo (Mihály et al., 2001; Szakács et al., 2003). In a previous investigation (Mihály et al., 2005), RT-PCR studies demonstrated the time-related changes of the c-*fos* mRNA in the cerebral cortex following 4-AP injection. Acute brief, repetitive seizures elicited by 4-AP led to rapid and transient c-*fos* mRNA expression in forebrain structures (Szakács et al., 2003; Mihály et al., 2005), resulting in marked increase of Fos protein immunoreactive cells by 30 min following the administration of 4-AP (Mihály et al., 2005). However, the c-*fos* mRNA displayed the first significant rise 60 min later, indicating that cytoplasmic Fos protein entered the neuronal cell nuclei at the time when c-*fos* mRNA levels were not yet elevated (Mihály et al., 2005). The seizure-activated intranuclear translocation of the Fos protein depends on extracellular signals (Roux et al., 1990), such as neuronal depolarization and Ca^{2+} influx. The stimulated translocation of the cytoplasmic Fos protein to the nucleus, where it binds to the AP-1 sequence (Herdegen and Leah,

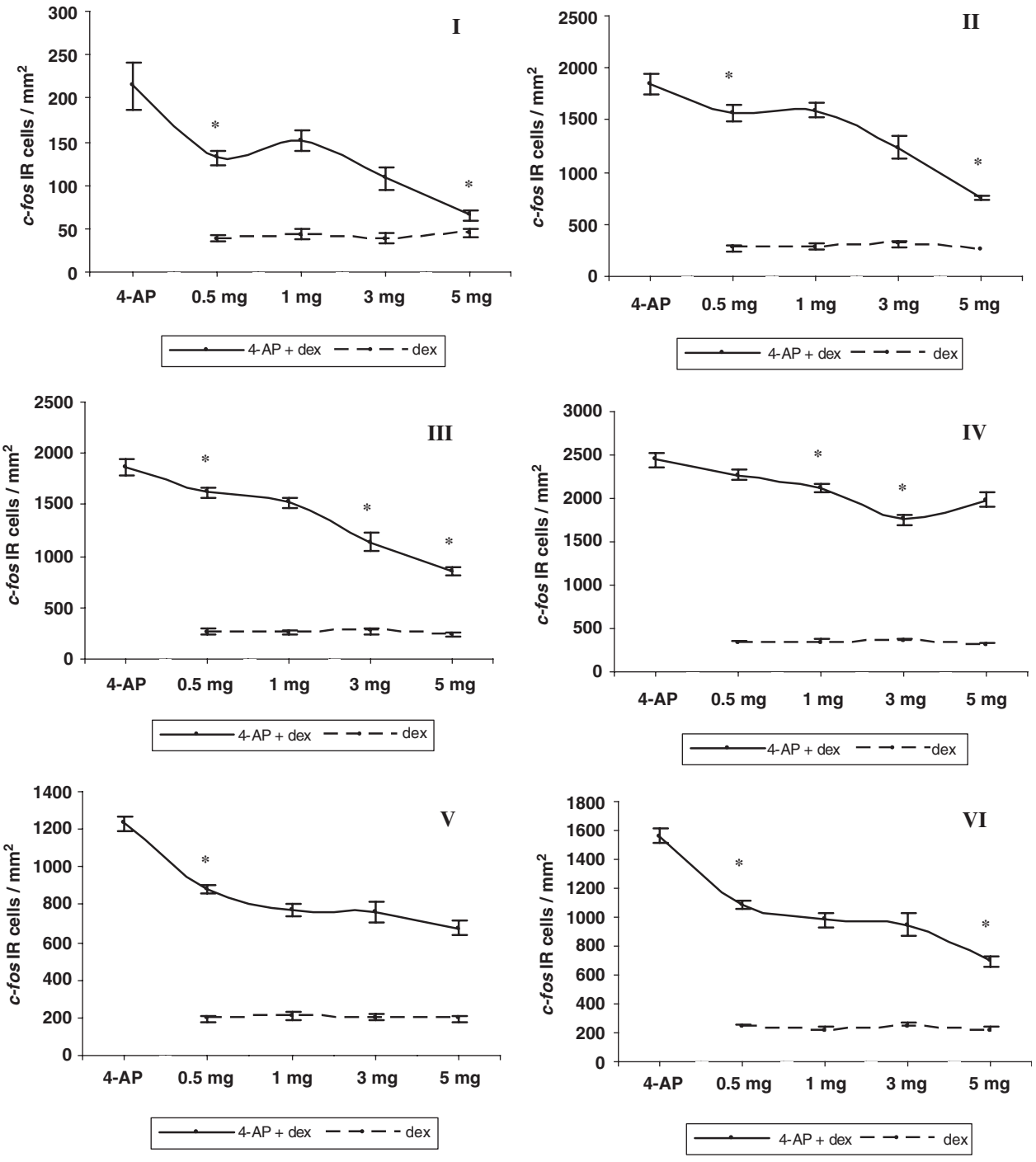

Figure 3. Analysis of the effects of dexamethasone on seizure-induced c-*fos* protein immunoreactivity in the different layers of the neocortex (Roman numerals denote neocortical layers). Continuous line: animals pretreated with dexamethasone and then with 4-AP (4-AP+dex). Dotted line: animals treated only with dexamethasone (dex). Vertical bars: S.E.M.; asterisks: significant differences ($p<0.05$; ANOVA, *post hoc* Bonferroni test). Abscissa: doses of dexamethasone; ordinate: c-*fos*-immunoreactive cell number per mm^2.

1998), will lead to a large induction of c-*fos* transcription, resulting in elevated c-*fos* mRNA levels for several hours (Mihály et al., 2005).

It is important to emphasize that pretreatment with dexamethasone reduced c-*fos* protein expression in the neocortex and hippocampus, and simultaneously provided symptomatic seizure protection. Our findings concerning seizure protection are in accord with literature data: dexamethasone pretreatment prevents μ- and δ-opioid

peptide-induced hippocampal seizures in vivo (Di Giannuario et al., 2001), and displays an antiepileptic effect in picrotoxin-elicited seizures on hippocampal slice culture (Duport et al., 1997). Moreover, it has been reported that the liposteroid dexamethasone palmitate could be effective for the treatment of refractory seizures in children, abolishing uncontrollable seizures and decreasing seizure frequency (Yoshikawa et al., 2000). The decrease of the number of c-*fos*-immunolabelled cell nuclei in the hippocampus reflects the distribution of glucocorticoid and mineralocorticoid receptors (Patel et al., 2000). These receptors (at the mRNA level) are strongly expressed in the *stratum pyramidale* of the Ammon's horn, and also the granule cell layer of the dentate fascia (Patel et al., 2000). Accordingly, we noted a strong decrease in c-*fos* immunolabelling in these regions. Dose dependency was not clear-cut, although the strongest effect was seen with 5 mg/kg dexamethasone. In the neocortex, receptor mRNAs were strongly expressed in layers II/III and V (Patel et al., 2000). Accordingly, we noted a strong, dose-related, significant and large decrease in c-*fos*-immunopositive cell number in these layers, although dose-dependency was not conspicuous in layer V.

While dexamethasone pretreatment significantly decreased the number of Fos-immunoreactive cell nuclei in the neocortex and the hippocampus, it did not decrease the boosted c-*fos* mRNA levels. We suggest that dexamethasone elicits its effect, in part, by suppressing intranuclear translocation of the Fos protein. Preventing translocation of the Fos protein to the nucleus will lead, therefore, to decreased number of Fos-positive cell nuclei. Literature data indicate that translocation of Fos protein from the cytoplasm to the nucleus depends on extracellular signals: neuronal depolarization and increase of intracellular Ca^{2+} levels stimulate this process (Mihály et al., 2005; Roux et al., 1990). According to the classical cellular mechanism of steroid action (Nair et al., 1998; Reagan and McEwen, 1997), dexamethasone is supposed to bind to intracellular receptors and induce genomic effects through new protein synthesis (Di Giannuario et al., 2001), which, in turn, can lead to the modulation of Ca^{2+} signals. In addition to these classical genomic effects of steroid action, it has been demonstrated that corticosterone significantly decreases NMDA receptor-mediated Ca^{2+} elevation in hippocampal slices (Sato et al., 2004), as well as in cultured neonatal hippocampal neurons (Takahashi et al., 2002). Furthermore, it has been shown that dexamethasone causes dissociation of the Raf-1/heat shock protein 90 (Hsp90) complex in cultured cells (Cissel and Beaven, 2000). A similar mechanism could be operative in the case of c-*fos* protein: dissociation of the Hsp/c-*fos* protein complex prevents the translocation to the nucleus. Therefore, we believe that dexamethasone can modulate intracellular Ca^{2+} signals via classical genomic pathways (through new protein synthesis, probably altering glutamate receptor subunit composition), and via non-genomic pathways (through NMDA receptor-mediated Ca^{2+} influx and - according to Pratt (1998) - by acting on cytosolic chaperones). These hypothetic explanations are to be tested in our laboratory in the near future.

The other possibility is that dexamethasone upregulates Fos ubiquitination and consequent degradation, thereby decreasing intracellular Fos protein levels. Literature data have shown such effects of dexamethasone on other nuclear regulatory proteins (Sundberg et al., 2006). Thus, we conclude that in our experiments the influence of dexamethasone on the expression of the c-*fos* protooncogene may be mediated by genomic and non-genomic mechanisms, presumably targeting the intranuclear translocation and/or the degradation of Fos protein. On the other hand, dexamethasone does not exert regulatory effects directly on the transcription of the c-*fos* gene.

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