NEURONAL PROTO-ONCOGENE EXPRESSION IN THE PHARMACOLOGICAL ASSESSMENT OF THE SYNAPTIC MECHANISMS OF THE HIPPOCAMPUS IN RATS

Réka Szakács, M.D.

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Department of Anatomy
and Department of Psychiatry
Faculty of Medicine, University of Szeged

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Animum rege, qui nisi paret, imperat.

(Horatius)

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ABSTRACTS RELATED TO THE THESIS

I. **Szakács R.**, Czigner A., Bohata Cs., Mihály A.: Time-dependent expression of c-fos protein in the rat forebrain following 4-aminopyridine seizures. *Cephal. Hung.* 1999; 5: P-195.

II. **Szakács R.**, Bohata Cs., Dobó E., Mihály A.: C-fos protein expression in the rat hippocampus following 4-aminopyridine seizures. *11th International Congress of Histochemistry and Cytochemistry (ICHC), University of York, UK,* 2000*;* Abstract Booklet: N-10.

III. **Szakács R.**, Bohata Cs., Dobó E., Mihály A.: C-fos protein expression in the rat hippocampus following 4-aminopyridine seizures. *Neurobiology* 2001; 8: 397-398.

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List of abbreviations:

5-HT	5-hydroxy-tryptamin, serotonin
AD	Alzheimer's disease
AGPC	acid guanidium thiocyanate-phenol-chloroform
AMPA	*α*-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ANOVA	analysis of variance
AOI	area of interest
AP-1	activator protein 1
4-AP	4-aminopyridine
AP5	2-amino-5-phosphonovaleric acid
AP7	2-amino-7-phosphonoheptanoic acid
CA	cornu Ammonis
cAMP	3',5'-cyclic adenosine monophosphate
c-*fos*	cellular proto-oncogene
CNS	central nervous system
CRE	cAMP response element
CREBP	cAMP response element binding protein
DAB	diaminobenzidine tetrahydrochloride
DG	dentate gyrus
DNA	deoxyribonucleic acid
EEG	electroencephalography
GABA	gamma aminobutyric acid
GAD	glutamic acid decarboxylase
GADPH	glyceraldehyde 3-phosphate dehydrogenase
GluR	glutamate receptor
GTCS	generalized tonic-clonic seizures
GTP	guanosine triphosphate
IgG	immunoglobulin G
LSD	lysergic acid diethylamide
LTD	long-term depression
LTP	long-term potentiation
mRNA	messenger ribonucleic acid
Ni-DAB	nickel-containing diaminobenzidine tetrahydrochloride
NMDA	*N*-methyl-D-aspartate
PAP	peroxidase-antiperoxidase
PBS	phosphate-buffered saline
PCP	phencyclidine
PCR	polymerase chain reaction
PV	parvalbumin
RT	reverse transcription
SEM	standard error of the mean

CONTENTS

1. INTRODUCTION

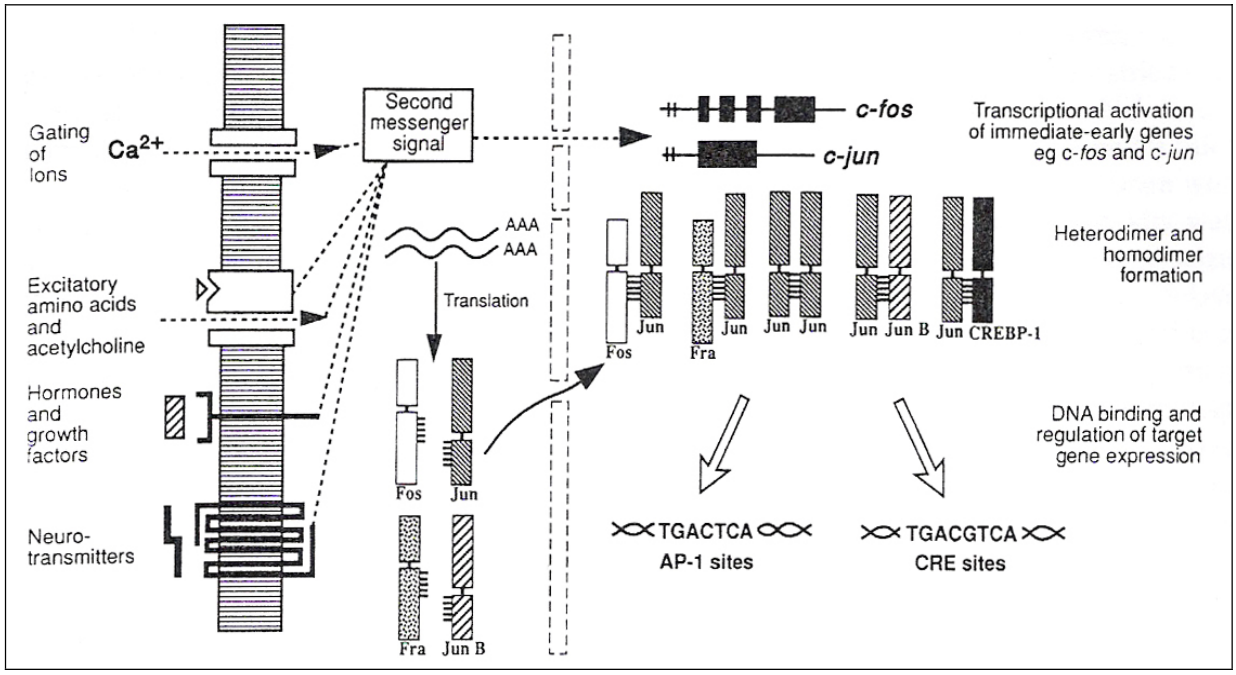
1.1. Proto-oncogenes in the nervous system

1.1.1. The c-fos proto-oncogene

Neuronal proto-oncogenes represent genetic elements that mediate both short- and long term changes in neuronal function. They are expressed in neural tissues and are subject to acute and long-term changes in expression and activity during signaling and cellular differentiation (Hanley et al., 1988). Signal transduction cascades, therefore, do not terminate in the cytoplasm but extend to the nucleus, leading to alterations in gene expression. Thus, proto-oncogenes constitute a signaling pathway which transfers information from the cell surface to the nucleus (Morgan & Curran, 1995). Several proto-oncogenes encode proteins that function as extracellular growth factors, membrane receptors, cytoplasmic and membrane-associated protein kinases, guanosin triphosphate (GTP)-binding proteins, and transcription factors. The c-*fos* protooncogene belongs to the inducible transcription factors (Herdegen & Leah, 1998), and exerts different regulatory actions in the cell nucleus. The c-*fos* gene is a member of immediate early genes (IEGs) that are activated rapidly by many extracellular stimuli, including neurotransmitters that enhance Ca^{2+} influx into the postsynaptic neuron (Greenberg & Ziff, 2001). The signals and intracellular pathways, leading to the expression of the c-*fos* gene, have generally been identified. Membrane depolarization induced by excitatory amino acids and acetylcholine, influx of Ca^{2+}, cAMP, hormones and some growth factors are able to influence c-*fos* expression, predominantly by means of intracellular protein kinase cascades. The activation of second messengers, protein kinases and other transcription factors leads to the accumulation of c-*fos* mRNA and the translocation of the synthesized Fos protein into the cell nucleus (Morgan & Curran, 1995; Figure 1). The c-*fos* gene-encoded Fos, a 55-62 kDa phosphorylated protein, forms a heterodimer with members of the Jun family of transcription factors. This association of Fos and Jun occurs through an α-helical domain containing a heptad repeat of leucine residues, termed leucine-zipper (Landshultz at al., 1988). Both Fos and Jun, as well as all members of the *fos* and *jun* gene families possess leucine zippers. Fos/Jun heterodimers bind to the consensus DNA sequence element, the AP-1 site. This sequence was identified as the binding site for the transcription factor activator protein 1 (AP-1), and is essential for basal and stimulated transcription from several genes (Curran & Franza, 1988). The AP-1 regulatory

proteins such as Fos, Fra and Jun, participate in the regulation of degeneration and regeneration in the developing and adult brain (Willoughby et al., 1995).

Figure 1. Extracellular signals and intracellular pathways leading to the transcriptional activation of the c-*fos* proto-oncogene. Membrane depolarization, influx of Ca^{2+}, hormones and some growth factors are able to influence c-*fos* expression, by second messenger signals. This leads to the accumulation of c-*fos* mRNA and the translocation of the synthesized Fos protein into the cell nucleus. Fos/Jun heterodimers bind to the AP-1 consensus site (whereas Jun-CREBP1 dimers bind at a CRE site).



CRE: cyclic adenosine monophosphate (cAMP) response element; CREBP: cyclic adenosine monophosphate (cAMP) response element binding protein

1.1.2. Induction of c-fos expression

Fos protein is normally not expressed in neurons, although there is a low level of Fos in some structures of the adult forebrain, but not in the hippocampus (Herdegen & Leah, 1998). Seizure activity induced by various types of convulsant agents leads to a rapid, massive and transient appearance of a series of IEG proteins such as Fos, throughout those brain regions that normally express low basal levels (Willoughby et al., 1995). The mRNA and protein are

detectable in the cerebral cortex and the hippocampus for a determined period following seizure activity (Gass et al., 1992; Zimmer et al., 1997; Willoughby et al., 1997). These investigations provide evidence that Fos protein is induced in response to strong synaptic activation. The postsynaptic c-*fos* mRNA expression correlates well with the presynaptic release of excitatory neurotransmitters (Labiner et al., 1993), therefore the immunohistochemical detection and evaluation of Fos protein appears suitable for the histological mapping of neuronal hyperactivity (Labiner et al., 1993; Mihály et al., 1997; Willoughby et al., 1997). Expression of c-*fos*, typically induced by epileptic seizures, is mediated by excitatory neurotransmitters acting on ionotropic receptors and the voltage-dependent Ca^{2+} channels in the postsynaptic membrane (Greenberg & Ziff, 2001). *In vitro* studies on the involvement of excitatory amino acid receptors have demonstrated that glutamate receptors of the *N*-methyl-D-aspartate (NMDA) and *α*-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) types, as well as the nicotinic acetylcholine receptor are able to activate c-*fos* expression (Herdegen & Leah, 1998). *In vivo*, the NMDA and AMPA receptors are the main candidates for c-*fos* induction (Greenberg & Ziff, 2001).

1.2. Mechanism of c-fos induction by 4-AP seizures

The compound 4-aminopyridine (4-AP) is a blocker of K^+ conductances, and specifically those of the K_A-channels, which regulate spike frequency in postsynaptic structures, and the K_V-channels, which are involved in the repolarisation phase of the action potential (Alexander & Peters, 2000). This prolonged action potential duration, the delay in neuronal repolarisation increases transmitter release and augments inhibitory and excitatory postsynaptic potentials in different CNS preparations *in vitro* (Tapia & Sitges 1982; Versteeg et al., 1995), and produces intense epileptiform activity in brain slices (Perrault & Avoli, 1991) and *in vivo* (Cramer et al, 1994). The enhanced presynaptic activity caused by 4-AP is reflected in the increased synaptic vesicle exocytosis at the ultrastructural level (Tokunaga et al., 1979). Additionally, 4-AP crosses the blood-brain barrier quickly and will be secreted into the cerebrospinal fluid and eliminated by the kidney (Lemeignan et al., 1984). In consequence of its fast action, the latency of the seizure induced by 4-AP is relatively short, and the convulsive activity extends probably to the whole forebrain (Mihály et al., 1990). *In vivo* studies indicated significant increases in regional cerebral blood flow in the neocortex, diencephalon and dentate gyrus in

mice following 4-AP injection (Mihály et al., 2000). 4-AP, therefore, is used for seizure induction both *in vivo* (Mihály et al., 2005) and *in vitro* (Brückner & Heinemann, 2000; Marinelli et al., 2000).

Focal and systemic administration of 4-AP induces rapidly an intense expression of c-*fos* in the neocortex and hippocampus (Mihály et al., 1997). Based on the mechanism of action of 4-AP, expression of c-*fos* is supposed to be mediated by transmitter actions: membrane depolarisation and Ca^{2+} influx. Literature data indicate that glutamate is the main candidate in the precipitation and maintenance of 4-AP seizures: enhancement of glutamatergic transmission has been related to the convulsant action of 4-AP (Tapia et al., 1999), since glutamate receptor antagonists of both the NMDA and non-NMDA types are effective anticonvulsants against 4-AP-induced seizures (Perrault & Avoli, 1991; Morales-Villagrán et al., 1996). 4-AP acts on several hippocampal pathways and augments the release of glutamate from their synapses. Microdialysis experiments proved that intrahippocampal perfusion of 4-AP produces intense electroencephalographic (EEG) seizures associated with neuronal damage in the CA1 and CA3 regions, which correlate well with the increase in the extracellular glutamate concentration in the hippocampus of rats (Peña & Tapia, 1999). Moreover, studies with microdialyis probes in the striatum of rats proved that 4-AP significantly increased glutamate level in the dialysis fluid, and the increase in the c-*fos* expression correlated well with the enhancement of glutamate release (Kovács et al., 2003). We consider, therefore, that 4-AP induces c-*fos* expression in part through increased release of glutamate from the cerebrocortical synapses *in vivo*, and in part through the concomitantly increased Ca^{2+} influx into the postsynaptic cell. Different glutamate receptors may play a role in the seizure-induced c-*fos* expression. The postsynaptic membranes of neocortical and hippocampal neurons possess not only NMDA, but also AMPA and kainate receptors (Löscher 1998). These ionotropic receptors respond to extracellular glutamate: AMPA receptors trigger depolarization and burst initiation, and NMDA receptors become then activated, leading to the opening of the NMDA channel which generates large Ca^{2+} influx (Hwa & Avoli, 1991; Löscher 1998). Blockade of the NMDA receptors inhibits the expression of c-*fos* mRNA in the dentate granule cells (Labiner et al., 1993), suggesting that neuronal hyperactivity and thereby c-*fos* induction are mediated by NMDA receptor activation. Accordingly, decreased seizure-induced c-*fos* expression following the administration of NMDA antagonists indicates weakening of the postsynaptic effects of glutamate and concomitant influx of Ca^{2+} (Greenberg & Ziff, 2001). Both NMDA and non-

NMDA glutamate receptors are known to contribute to the ictagenic effects of 4-AP *in vitro* (Gean, 1990). Studies involving intracellular recording in rat neocortical slices have proved that NMDA receptors contribute to the process of stimulus-induced paroxysmal depolarization shift amplification by prolonging the duration and reducing the latency of the epileptiform discharge (Hwa & Avoli, 1991).

1.3. Neurotransmission in the hippocampus

1.3.1. Glutamatergic neurotransmission – structural and functional implications

Glutamate causes a marked increase in the firing rate of granule and pyramidal cells (Baudry & Lynch, 1981) and is the major excitatory transmitter in the hippocampus (Frotscher et al., 1988). The main glutamatergic afferents to the hippocampus are represented by the perforanth path originating in the entorhinal cortex and terminating in the molecular layer of the dentate gyrus and in the stratum lacunosum-moleculare of the Ammon's horn. The dentate gyrus is the target for the majority of entorhinal afferents by providing sensory information of multiple modalities about the external world (Freund & Buzsáki, 1996). Afferents from the entorhinal cortex terminate in the molecular layer on dendrites of predominant granule cells. The dentate gyrus is considered to be the first element in the hippocampal trisynaptic chain that additionally includes the synapses between mossy fibers and CA3 pyramidal cells, and synapses of the Schaffer collaterals with CA1 pyramidal cells (Frotscher et al., 1988; Freund & Buzsáki, 1996). The mossy fibers arise from the dentate granule cells and provide synaptic input to the neurons of the hilus and to CA3 area of the hippocampus (Henze et al., 2000). The mossy fiber input to CA3 area comprises, therefore, the second synapse of the trisynaptic circuit, the pyramidal cells of this regions being the principal targets of granule cell axons. CA3 pyramidal cells also receive a direct projection from the entorhinal cortex, which terminates on dendrites in stratum lacunosum-moleculare (Amaral & Witter, 1989). It is, however, important to emphasize that mossy fiber collaterals form synapses in the hilus with a great number of inhibitory interneurons (Henze et al., 2000), and also provide innervation to excitatory, glutamatergic hilar mossy cells (Soriano & Frotscher, 1994). These inhibitory hilar interneurons provide GABAergic input to CA3 pyramidal cells and thus lead to feedforward inhibition following mossy fiber activity (Vida

& Frotscher, 2000). Activation of the mossy fiber pathway results in the activity of greater number of inhibitory interneurons than excitatory hilar mossy cells and CA3 pyramidal cells, which causes the activation of a very specific subset of CA3 pyramidal cells (Henze et al., 2000). CA1 area is the major target of CA3 pyramidal cell axons, the Schaffer collaterals, and the pyramidal cells of this subfield represent the third and last synaptic connection in the classical hippocampal trisynaptic chain. There is extensive literature evidence that the neurotransmitter for members of this trisynaptic circuit is glutamate (Frotscher et al., 1988). Glutamate, as the major excitatory transmitter in the hippocampus, is involved in long-term potentiation (LTP) (Bliss & Lomo, 1973), synaptic plasticity (McEwen, 2001), epileptic seizures (Peña & Tapia, 2000), excitotoxicity and neurodegeneration (Olney, 1990). One postulated mechanism for cellular events involved in the rapid formation of new memories is LTP, which is a property of certain synapses, including those of the mossy fibers and the Schaffer collaterals of the hippocampus. LTP means the increase in synaptic efficiency and, therefore, high-frequency activity of the presynaptic terminals leads to increased activity of affected postsynaptic neurons. A suitable pattern of afferent connections of the hippocampus may lead to LTP in certain granule and pyramidal cells, which will continue to transmit impulses more frequently, even though the external stimulus has ceased (Bliss & Lomo, 1973). Long-term alteration of synaptic transmission occurs at excitatory synapses, which is thought to be the basis of specific associative memory. However, experiments suggest that GABAergic synapses on principal cells and interneurons may also undergo long-term modifications (Marty and Llano, 1995; Freund & Buzsáki, 1996). *In vitro* studies on LTP of interneurons indicate that, similar to LTP of principal cells of the hippocampus, a possible site of modification is the excitatory synapses on interneurons (Ouardouz & Lacaille, 1995). Both LTP and long-term depression (LTD) depend on activity-dependent Ca^{2+} increase in the postsynaptic neurons, and enhancement or decrease in the LTP/LTD phenomenon is the cellular basis of synaptic plasticity. Synaptic plasticity depends, therefore, on the increase of intracellular Ca^{2+} level in the postsynaptic neuron, which occurs mainly through the glutamate receptors of the NMDA subtype and the voltage-dependent Ca^{2+} channels (Freund, 1999). In particular, glutamate and NMDA receptors play an important role in hippocampal functional changes and structural plasticity, including synapse formation in CA1 region (McEwen, 2001; Woolley et al., 1997), regulation of adult neurogenesis of dentate gyrus granule cells (Cameron et al., 1995; Gould & Tanapat, 1999) or remodeling of apical dendrites in CA3 area (McEwen, 2001).

1.3.2. NMDA receptor in the hippocampus – relevance to neuropsychiatric disorders

Studies of the hippocampus focusing on the excitatory amino acid glutamate have revealed the central role of this compound in both the normal and abnormal functioning of this brain structure. Extensive research on the hippocampus as a target of stress and neurotransmitters indicates that the NMDA subtype of the ionotropic glutamate receptor family is the most frequently implicated in functional and structural changes seen in several neuropsychiatric disorders (Olney 1990; McEwen, 2001). Different modalities of stress affect hippocampal structural plasticity, including suppression of the ongoing neurogenesis in the dentate granule cells, through an NMDA-receptor-mediated excitatory pathway (Gould & Tanapat, 1999). Excitatory input to the granule cell population from the entorhinal cortex acts via NMDA receptors, regulating along with circulating adrenal steroids the rate of neurogenesis and apoptotic cell death, and both acute and chronic stress appear to inhibit neurogenesis in the dentate gyrus (McEwen, 2001). In adulthood, granule cell neurogenesis, as well as apoptotic neuronal cell death, therefore, are regulated by stress, as well as by seizure activity. Accordingly, in animal experiments blockade of NMDA receptors enhances granule cell proliferation, whereas activation of NMDA receptors inhibits cell proliferation in the dentate gyrus (Gould & Tanapat, 1999). Conversely to the stress-induced activation of NMDA receptors, which decreases neurogenesis in the dentate gyrus, serotonin (5-HT) stimulates neurogenesis of granule cells (Gould, 1999), via activation of the 5-HT1A receptors. This reveals the possibility that the inhibitory effects of stress on granule cell production may be prevented by 5-HT1A receptor agonists, including administration of several classes of antidepressants (Duman, 1999). Jacobs et al. assumed that stress-induced decreases in dentate gyrus neurogenesis (via NMDA glutamate receptors) might be an important causal factor in precipitating clinical depression, and, thereby, therapeutic interventions for depression that increase serotonergic neurotransmission act in part by augmenting dentate neurogenesis (Jacobs et al., 2000). Moreover, glutamate release during repeated stress, including chronic psychosocial stress, leads to atrophy of apical dendrites in the CA3 region of hippocampus, possibly through NMDA receptors and reduced GABA-mediated inhibitory activity at synapses from the interneurons on CA3 pyramidal neurons. This process that causes remodeling of dendrites in the CA3 region, results in cognitive

impairment in the learning of spatial and short-term memory tasks (Magarinos et al., 1996; McEwen, 1999). Investigation of the process of dendritic atrophy in the hippocampus might reveal some of the cellular mechanisms of human hippocampal atrophy seen in particular in recurrent depression, Cushing's syndrome or post-traumatic stress disorder (PTSD) (Szakács & Janka, 2002). It is, however, important to mention, that hippocampal atrophy and accompanying cognitive impairment seen in schizophrenia and Alzheimer's disease (AD) arises from *under*excitation of NMDA receptors on inhibitory neurons. Glutamate acting through NMDA receptors on GABAergic, serotonergic and noradrenergic neurons maintains tonic inhibitory control over excitatory pathways that innervate corticolimbic pyramidal cells. Mechanisms, including genetic factors and early brain insults in schizophrenia (Lyon et al., 1989; Olney & Farber, 1995), or predisposing factors (e.g. apoE4 genotype in sporadic AD, amyloidogenic mutations in familial AD) which promote amyloidosis in AD (Patel, 1995) lead to the blockade of these NMDA receptors/or loss of NMDA receptor-bearing inhibitory neurons. Loss of these inhibitory neurons and/or their NMDA receptors will, in turn, abolish inhibitory control over excitatory inputs on corticolimbic pyramidal cells, creating disruption among multiple intracellular second messenger systems, and thereby causing impairment of cognitive functions, as well as degeneration of the afflicted neurons (Farber et al., 1998; Bressan & Pilowsky, 2000).

Glutamate and NMDA receptors are also involved in neuronal death in pyramidal cells of the hippocampus following seizures and ischaemia. The hippocampal CA1 pyramidal cells are exceptionally sensitive to hypoxia and are among the first to be affected in conditions that lead to oxygen deprivation. Excessive activation of NMDA receptors (NMDA hyperfunction) plays an important role in the pathophysiology of acute injury syndromes such as hypoxia-ischaemia, trauma and status epilepticus (Olney, 1990). Given the role of glutamate in epilepsy, and the apparent neurotoxicity of NMDA receptor activation, different glutamatergic parameters of seizure activity have been evaluated, including altered expression of NMDA receptors, as well as anticonvulsant and neuroprotective effects of NMDA antagonists. Interestingly, elimination of the NR1 subunit of the NMDA receptor (NMDAR1 knockouts) in the hippocampal CA1 region in transgenic mice leads to impaired plasticity and learning, but no seizures in this particular hippocampal domain (Wilson & Tonegawa, 1997). Considering the involvement of NMDA receptors in synaptic plasticity, seizures and neurotoxicity, a number of NMDA antagonists have been evaluated for therapeutic purposes. There is an extensive literature evidence for the anticonvulsant and neuroprotective properties

of glutamate receptor antagonist drugs in various *in vivo* and *in vitro* epilepsy models (Mares et al., 1992; Patel et al., 1990;, Rogawski et al., 1991). The competitive and selective NMDA antagonists, 2-amino-5-phosphonovaleric acid (AP5) and 2-amino-7-phophonoheptanoic acid (AP7) have been shown to be potent antiepileptic agents when given intracerebroventricularly (i.c.v.) to mice (Croucher et al., 1982; Meldrum et al., 1983). Competitive NMDA antagonists such as D-CPP and D-CPPene are particularly effective against reflex epilepsy in rodent and primate epilepsy model, without apparent motor or sedative side effects (Patel, 1990). Competitive NMDA antagonists are also effective against chemically-induced seizures and maximal electroshock. High-affinity open-channel NMDA receptor blockers such as phencyclidine (PCP), ketamine and dizocilpine (MK-801) are also potent anticonvulsants (Apland & Cann, 1995; Veliskova et al., 1990), and protect against seizure-related brain damage (Cifford et al., 1989, 1990). Low-affinity open-channel NMDA receptor blockers such as amantadine (1-aminoadamantane), memantine (1-amino-3,5-dimethyladamantane), remacemide (and its desglycinated metabolite), dextrometorphan ((±)-3-methoxy-*N*-methylmorphinan) and its metabolite dextrorphan also display anticonvulsant and neuroprotective activities (Rogawski, 1992; Sagratella, 1995; Parsons et al., 1995; Hironaka & Niki, 2000). Some of these NMDA antagonist drugs are already in clinical use.

2. OBJECTIVES

I. The aim of our studies was to describe the distribution and the rate of appearance of the activated neurons in the hippocampus following 4-AP administration, using the immunohistochemical detection of Fos protein as a marker of neuronal activation.

II. We wanted to collect data on the participation of inhibitory neurons in the seizure process, therefore we used double immunolabeling to identify Fos-containing parvalbumin (PV)-positive interneurons in the hippocampus.

III. An objective was to test the changes in c-*fos* expression in the 4-AP seizure model following pretreatment with NMDA receptor antagonists, and thereby estimate the contribution of NMDA receptors to the seizure process.

3. MATERIALS AND METHODS

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). They were approved by the Faculty Ethical Committee on Animal Experiments, University of Szeged.

3.1. Animals handling in the 4-AP seizure model

The experiments were performed on male Wistar rats weighing 180-200 g.
The convulsant agent 4-AP (Sigma, St. Louis, MO) was dissolved in physiological saline (0.67 mg of 4-AP in 1 ml of solvent) and administered intraperitoneally (5 mg/kg b.w.) to 12 animals. This dose proved to be epileptogenic in previous investigations (Mihály et al., 1990, 2000). The control group (three animals) received the solvent of 4-AP (0,9 % sodium chloride in distilled water). The i.p. administration of 4-AP caused symptoms of generalized tonic-clonic seizures within 15 min in every treated animal. At the end of the experiment, 1, 3, and 5 h respectively after the injection of 4-AP (four animals from each, plus one control), the animals were deeply anaesthetized with diethyl ether and perfused transcardially with 500 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4).

The brains were removed, postfixed in 4% paraformaldehyde for 1 h at room temperature, and then cryoprotected overnight in 30% sucrose in 0.1 M phosphate buffer (pH 7.4). Serial frozen coronal plane sections were cut on a cryostat at a thickness of 24 μm, and every sixth section was processed for immunohistochemistry.

3.2. C-*fos* immunohistochemistry

Polyclonal c-*fos* antibody (raised in rabbit; Santa Cruz Biotechnology, CA) and the peroxidase-antiperoxidase (PAP) method were used. The sections were pretreated with 1.5% H_2O_2 and rinsed in 0.1 M PBS containing 0.2% Triton X-100. They were then incubated in 20% normal pig serum, next in primary c-*fos* antibody (1: 1000 in 20% normal pig serum in

PBS and 0.2% sodium azide), followed by donkey anti-rabbit IgG (1:40; Jackson ImmunoResearch, PA). The secondary antibody was detected by the PAP technique (PAP complex diluted to 1:1000), and the peroxidase reaction was localized with nickel-intensified diaminobenzidine tetrahydrochloride (Ni-DAB; Sigma, St. Louis, MO), yielding a black reaction product.

3.3. Parvalbumin (PV) and Fos double immunostaining

Primary antibody cocktails (two primary antibodies: mouse anti-PV, 1:100 000, and rabbit anti-c-*fos*, 1:1000) were used. The anti-parvalbumin serum was purchased from Sigma (St. Louis, MO). Primary antibodies were followed by biotinylated anti-mouse IgG (1:600; Vector Laboratories, CA) and plain donkey-anti-rabbit IgG (1:40; Jackson ImmunoResearch, PA), detected by streptavidin-peroxidase (1:2000; Vector Laboratories, CA) and PAP (1:1000), respectively. The streptavidin-peroxidase was developed by using plain diaminobenzidine tetrahydrochloride (DAB)

3.4. Animals and treatment in the pharmacological assessment of NMDA receptor antagonists

Experiments were performed on male Wistar rats weighing 180-200 g. The 4-AP was dissolved in saline (0.67 mg in 1 ml vehicle) and administered i.p. (5 mg/kg b.w.), as described above.

Pretreatments with ketamine (3 mg/kg b.w.), MK-801 (1 mg/kg b.w.), amantadine (40 mg/kg b.w.) or dextrometorphan (40 mg/kg b.w.) were performed in four groups, each containing three animals (12 animals). All tested drugs were purchased from Sigma (St. Louis, MO). The drugs were dissolved in saline and injected i.p. in a volume of 1 ml, 10 min prior to the application of 4-AP. One control group (three animals) received the same amount of solvent (0.9% sodium chloride in distilled water) and 4-AP (5 mg/kg b.w.). Other control groups received only the tested drugs, without 4-AP (12 animals). Finally, an additional control group (three animals) received only physiological saline. All of the above animal groups (30 rats in total) were used for immunohistochemistry. Three hours after the

administration of 4-AP, the animals were deeply anaesthetized with diethyl ether and perfused through the heart with 200 ml of 0.1 M PBS (pH 7.4), followed by 300 ml of 4% phosphate-buffered paraformaldehyde (pH 7.4).

As described above, the brains were removed, postfixed in 4% paraformaldehyde for 1 h at room temperature, and then cryoprotected overnight (30% sucrose in 0.1 M PBS, pH 7.4) at room temperature. Serial frozen sections were cut on cryostat in the coronal plane at a thickness of 24 μm, and every third section was then processed for immunohistochemistry.

The behavioural outcome of the pretreatment with NMDA receptor antagonist drugs, and in particular the latency of the onset of generalized tonic-clonic seizures from the time of 4-AP injection, was evaluated in parallel experiments, in groups of 15 animals each (75 animals in total; Table 1).

Table 1.

Behavioural analysis of the effect of NMDA antagonists on 4-AP seizures

Compound/s	GTCS Latency (min)	SEM	Animals Displaying GTCS (%)
4-AP	30.3	1.4	100
4-AP + amantadine	26.2	2.0	100
4-AP + dextrometorphan	45.0*	4.3	83.3
4-AP + ketamine	45.3*	4.7	61.1
4-AP + MK-801	34.2	4.5	27.7

The tests were conducted in groups of 15 animals each. The antagonists were injected i.p. Ten minutes later, 4-AP was administered, and the latencies of the onset of GTCS were measured from the time of the 4-AP injection. Significant differences are indicated. Abbreviations: GTCS: generalized tonic-clonic seizures; SEM: standard error of the mean.
* $p < 0.0.5$; ANOVA followed by the *post hoc* Bonferroni test.

3.5. C-*fos* mRNA detection

One hour following administration of 4-AP (5 mg/kg b.w.) and saline injection, respectively, the rats were decapitated under anaesthesia with diethyl ether (three animals in each group). Animals treated with 4-AP displayed behavioural seizures. The brains were dissected and samples of the parietal cortex were frozen in liquid nitrogen. Tissue samples

were homogenized and the total RNA was extracted by the AGPC method (Chomczynski & Sacchi, 1987). The reverse transcription (RT) was made from 2 μg RNA (Zádor et al., 1996). One μl of the RT product was submitted to multiplex polymerase chain reaction (PCR) in 50 μl volume of Taq reaction buffer containing 0.25 μM glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers, 2.5 μM c-*fos* primers, 200 μM dNTP, 1.5 mM $MgCl_2$ and 1 unit of Taq DNA polymerase. The sequence of GAPDH primers and the sequence of c-*fos* primers have been described (Zádor et al., 1996; Arrieta et al., 2000). 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 (377 bp) was confirmed by cloning into pGEM –T easy vector and sequencing. All chemicals were purchased from Sigma. 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 the ScanPack 10.1 A20 program (Biometra, Göttingen, Germany). The paired Student's *t*-test was used for statistical analysis. The levels of c-*fos* transcript in each of the samples were normalized to the level of GAPDH mRNA detected from the same amplification reaction.

3.6. Image analysis techniques

Quantitative analysis was performed on five histological sections from each animal, selected from every brain on the basis of the same stereotaxic coordinates (Paxinos & Watson, 1998). Areas of interest (AOIs) for counting immunostained cell nuclei were selected from regions CA1, CA2 and CA3 of the Ammon's horn, and from the hilus and granule cell layer of the dentate gyrus. In the pharmacological assessment of NMDA antagonists, we counted immunostained neuronal nuclei selected from the S1Tr region of the neocortex, as well.

The immunoreactive cell nuclei displaying grayish black staining were counted within each AOI with the aid of a Nikon Eclipse 600 microscope equipped with a SPOT RT Slider digital camera (1600 x 1200 dpi in 8 bits), using the Image Pro Plus 4 morphometry software (Media Cybernetics, Silver Spring, MD). Following background subtraction, the threshold was adjusted so that all labeled nuclei could be equally recognized. The AOIs were determined using the rectangular image-capturing field of the camera.

In the hippocampus, cell counts were done using a 40x objective, and in regions CA1-CA3, the AOI (an area of 0.05 mm^2) included the stratum pyramidale and a narrow zone of the strata oriens and radiatum. Cell counts were then normalized to 1 mm^2 tissue area. The hilus of the dentate gyrus was outlined according to Amaral (Amaral, 1978), and counting was performed. The whole extent of the upper and lower blades of the granule cell layer was outlined and used as AOI, and labeled neuronal nuclei were counted in this area. The molecular layer of the dentate gyrus contained very few Fos-positive cell nuclei, therefore was not evaluated quantitatively.

The PV-Fos double immunostaining was investigated in three animals. On double-stained histological sections, the neurons containing *Fos plus PV* or *PV only* were counted separately, the area of the hippocampus was then measured, and the cell counts were related to the hippocampal area in mm^2. The AOI in this case was determined manually, by labeling the outlines of the hippocampal formation.

The data were analysed statistically comparing sets of findings obtained with the same magnification. The cell counts were analysed by ANOVA, followed by the Bonferroni *post hoc* test. The statistical analysis was performed with the SPSS 9.0 software.

4. RESULTS

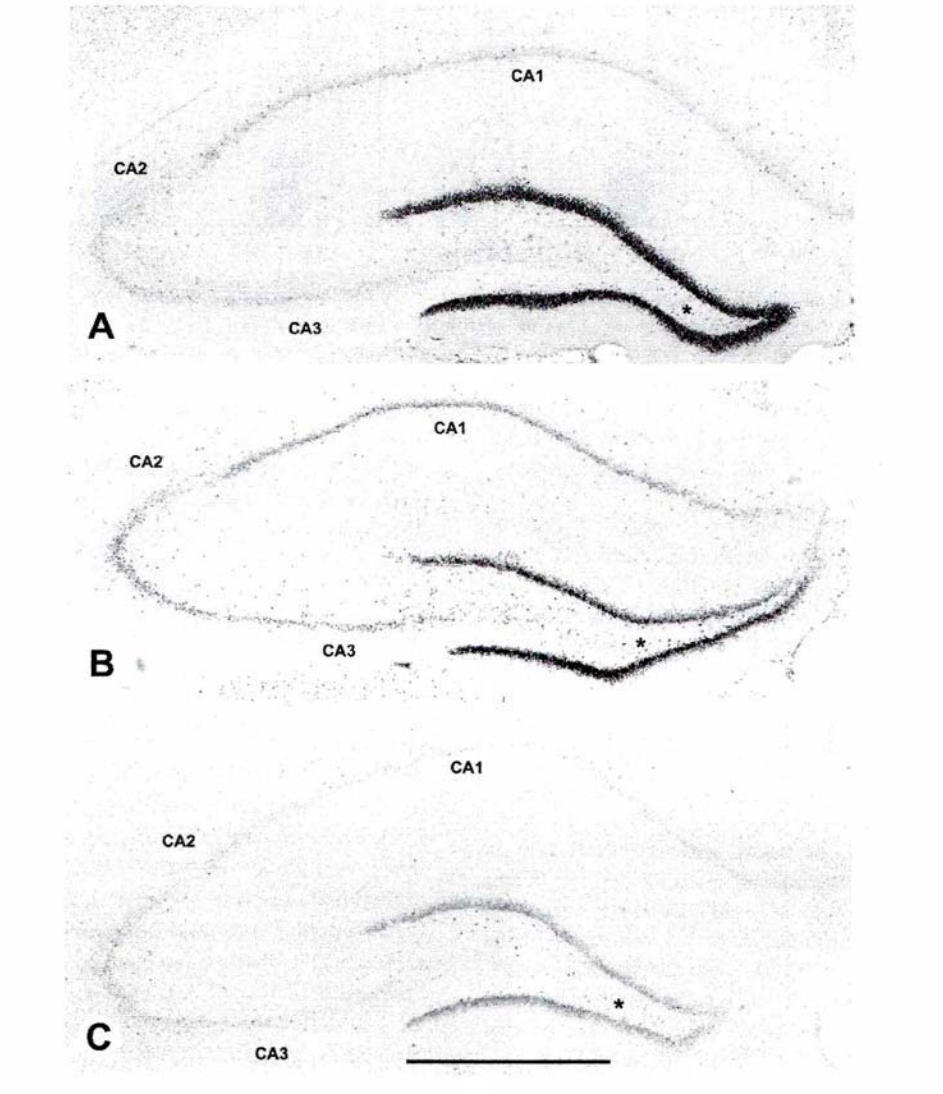
The i.p. administration of 4-AP caused characteristic behavioural symptoms within 15 min: increased exploratory activity, followed by tremor of the vibrissal and masticatory muscles, then generalized tremor of the body musculature, detectable as continuous fasciculation of the muscles, and finally generalized tonic-clonic seizures (GTCS). The symptoms of the GTCS were always sudden and clear-cut, therefore the latencies of the GTCS onset were easily measurable (average: 30.3 min).

4.1. Localization of Fos immunoreactivity in the 4-AP seizure model

Fos-positive cell nuclei were detected in the entorhinal and piriform cortices, the hippocampus, the lateral septum, the thalamus, the hypothalamus and in every layer of the neocortex. A few scattered nuclei were stained in the midline nuclei of the thalamus and

hypothalamus, and the dorsal striatum, whilst the ventral striatum was regularly devoid of immunostaining. Fos immunoreactivity was absent from these brain structures (including the hippocampal formation) in the control animals. In present study, we analysed the Fos-containing cell nuclei in the hippocampus 1, 3, and 5 h following the injection of 4-AP (Figure 2).

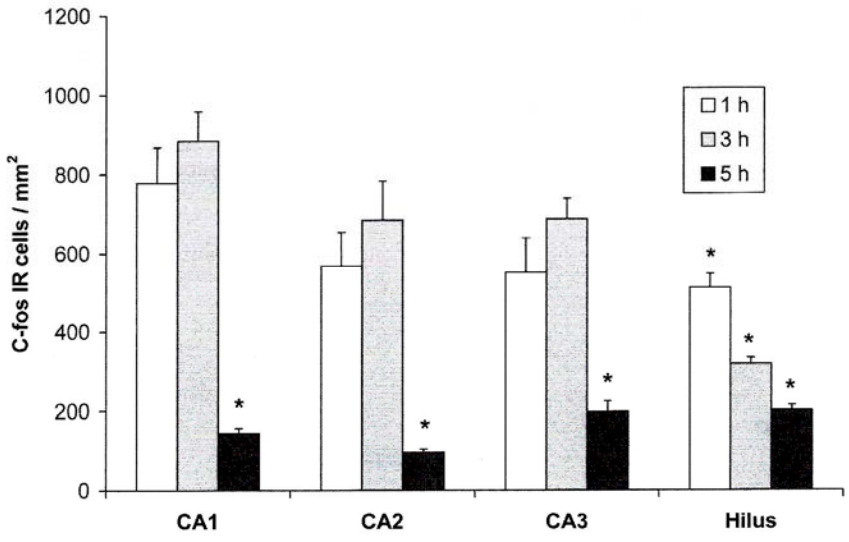
Figure 2. Low magnification images of the distribution of Fos-positive cell nuclei in the hippocampus at 1 h (A), 3 h (B), and 5 h (C) post-injection.



The sectors of Ammon's horn (CA1, CA2, CA3) are indicated. The asterisk shows the hilus of the dentate gyrus. Strong immunoreactivity of the granule cell layer can be observed at 1 h, which gradually decreased at 3 and 5 h. The staining intensity of the pyramidal cell layer of regions CA1-3 is strongest at 3 h. Bar: 1mm.

One hour after the injection, the granule cell layer of the dentate gyrus displayed very strong staining because of the high packing density of the Fos-immunoreactive granule cells (Figure 2A). The intensity of the immunostaining was so strong that counting of the activated cell nuclei was not possible at 1 h. Fos expression in the granule cell layer of the dentate gyrus decreased gradually between 1 and 3 h and was strongly reduced by 5 h following the injection of 4-AP (Figure 2A-C). Some scattered Fos-positive cell nuclei were seen in the molecular layer of the dentate gyrus, whilst the hilus contained many more activated cells. We therefore did not attempt to count the cell nuclei in the molecular layer, but we counted the Fos-containing cell nuclei in the hilus of the dentate gyrus. Similarly to the Fos expression in the granule cell layer, the number of Fos-positive cell nuclei in the hilus was highest at 1 h, and subsequently gradually decreased (Figure 3).

Figure 3. Time-dependent changes of Fos-positive cell counts in the Ammon's horn (CA1, CA2, CA3) and the hilus of the dentate gyrus (n = 15 in every case).



Significant differences are shown by asterisks (p = 0.001 in every case, except for the difference between the 3- and 5 h measurements of the hilus, where p = 0.003). The standard error of the mean (SEM.) is displayed on the top of the columns. (ANOVA, *post hoc* Bonferroni test)

The Ammon's horn apparently contained few Fos-labelled cells at 1 h post-injection. Immunostained nuclei were detected in the pyramidal cell layer of regions CA1, CA2 and CA3. CA2 and CA3 area regularly contained fewer Fos-immunoreactive cell nuclei than CA1. Apart from the pyramidal cell layer, few scattered cell nuclei were stained in the strata oriens, radiatum and lacunosum-moleculare. The number and staining intensity were increased at 3 h post-injection (Figure 2B). Regions CA1-3 of the hippocampus displayed strong Fos-like staining, mainly in the pyramidal layer. Fewer scattered cell nuclei were seen in the strata oriens, radiatum and lacunosum-moleculare. The counts for sectors CA1, CA2 and CA3 of the Ammon's horn supported these observations: the number of Fos-positive cell nuclei in these areas increased between 1 and 3 h following the injection and significantly decreased by 5 h (Figure 3).

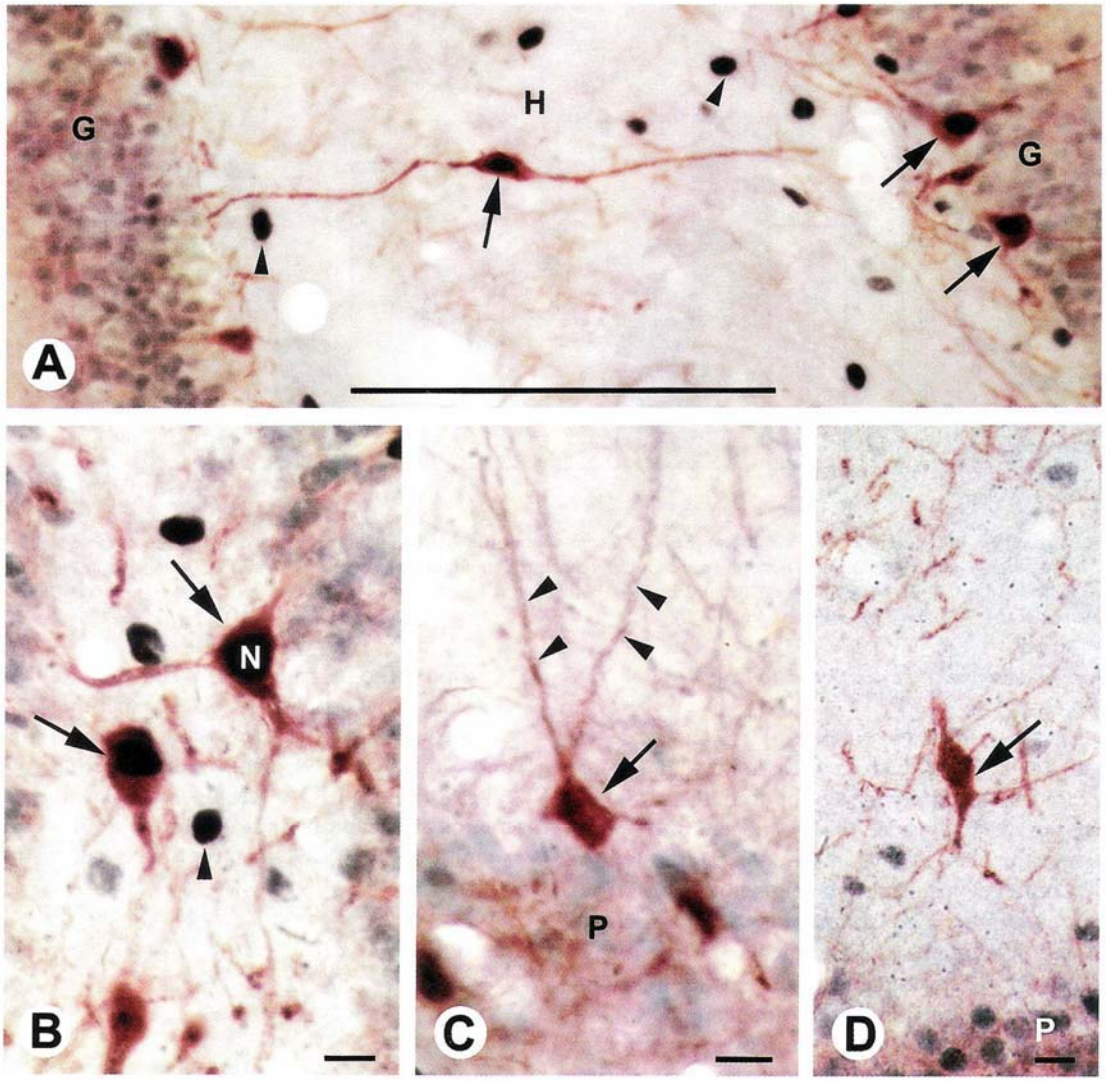
Quantitation therefore revealed that 3 h exposure-time to 4-AP was within the interval in which the Fos-containing cell nuclei could be identified in the dentate granule cell layer, and were maximal in the sectors of Ammon's horn (CA1, CA2 and CA3).

4.2. Parvalbumin localization in the rat hippocampus

PV-immunoreactive cell bodies were located principally in the pyramidal layer of the Ammon's horn, and in the hilus of the dentate gyrus. Some scattered PV-positive neurons were found in the strata oriens and radiatum of the Ammon's horn. The dendrites of the PV neurons were located mainly in the stratum radiatum and lacunosum-moleculare. A dense PV-stained fiber plexus was observed in the stratum pyramidale. In the dentate gyrus, most of the PV-positive cell bodies were seen in the hilus. Apart from the hilus, a few scattered cells were found in the granule cell layer and in the molecular layer. Most of the cells possessed long, thick, strongly stained dendrites. PV-Fos double immunostaining was clearly visible as Fos nuclear staining was displayed in black, gray and bluish black, whilst PV immunoreactivity was brown (Figure 4A-D).

The number of PV-positive neurons expressing Fos protein (PV-Fos neurons) in the Ammon's horn (CA) and in the dentate gyrus increased between 1 and 3 h, and then was strongly reduced by 5 h post-injection.

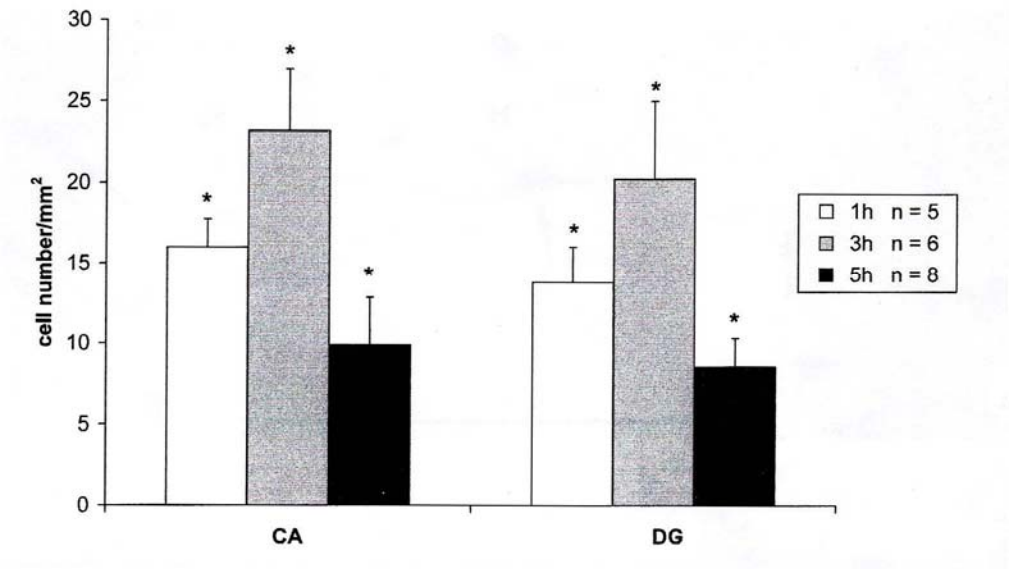
Figure 4. Appearance of the PV-Fos double immunostaining in the dentate fascia (A, B), sector CA2 (C), sector CA3 (D). Brown structures are PV-immunoreactive, while black and gray cell nuclei indicate Fos immunoreactivity.



(A) PV-positive neurons expressing Fos protein (arrows) in the dentate fascia at 3 h post-injection. Arrowheads point to Fos-stained cells of unknown identity. H: hilus; G: granule cell layer containing Fos-labelled nuclei. Bar: 100 μm. (B) Double-stained neurons (arrows) in the hilus at 3 h post-injection. The presence of Fos-immunoreactive nuclei (N) in the PV-positive neurons is clearly observable. The arrowhead points to the PV-negative Fos-containing cell. Bar: 10 μm. (C) PV-positive neuron (arrow) in the stratum radiatum of CA2 at 5 h post-injection. The neuron does not express Fos protein. Arrowheads point to the dendrites of the cell. P: pyramidal cell layer. Bar: 10 μm. (D) PV-positive neuron without Fos immunoreactivity (arrow) in the stratum lacunosum-moleculare of CA3, at 5 h post-injection. P: pyramidal cell layer containing Fos-stained cell nuclei. Bar: 10 μm.

We counted the activated PV cells related to the overall area of the hippocampal formation (Ammon's horn plus dentate gyrus). Significant differences were found between the 1- and 3 h counts, and between the 3- and 5 h counts. The number of PV-Fos neurons was highest at 3 h (Figure 5).

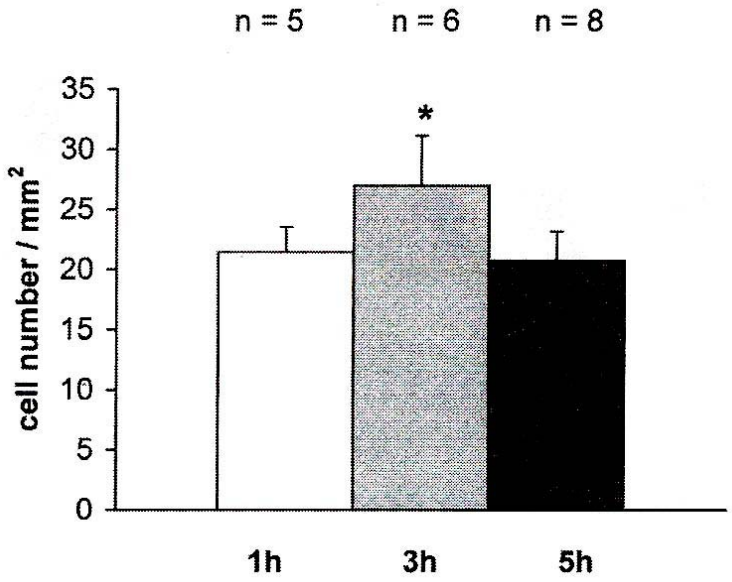
Figure 5. Changes in the number of PV-positive cells expressing Fos protein in the Ammon's horn (CA) and in the dentate gyrus (DG), at 1, 3 and 5 h post-injection.



Asterisks denote significant differences; S.E.M. and the number of measurements are indicated (in CA: $p = 0.004$ between 1 and 3 h; $p = 0.008$ between 1 and 5 h; $p = 0.001$ between 3 and 5 h; in DG: $p = 0.011$ between 1 and 3 h; $p = 0.001$ between 3 and 5 h). (ANOVA, *post hoc* Bonferroni test)

When we investigated the *total* number of PV-stained cells (PV-Fos neurons and PV neurons without Fos immunoreactivity), we found a slight increase in their numbers between 1 and 3 h, and a significant decrease between the 3- and 5 h counts (Figure 6).

Figure 6. Changes in the number of PV-positive neurons in the hippocampal formation (Ammon's horn plus dentate gyrus).

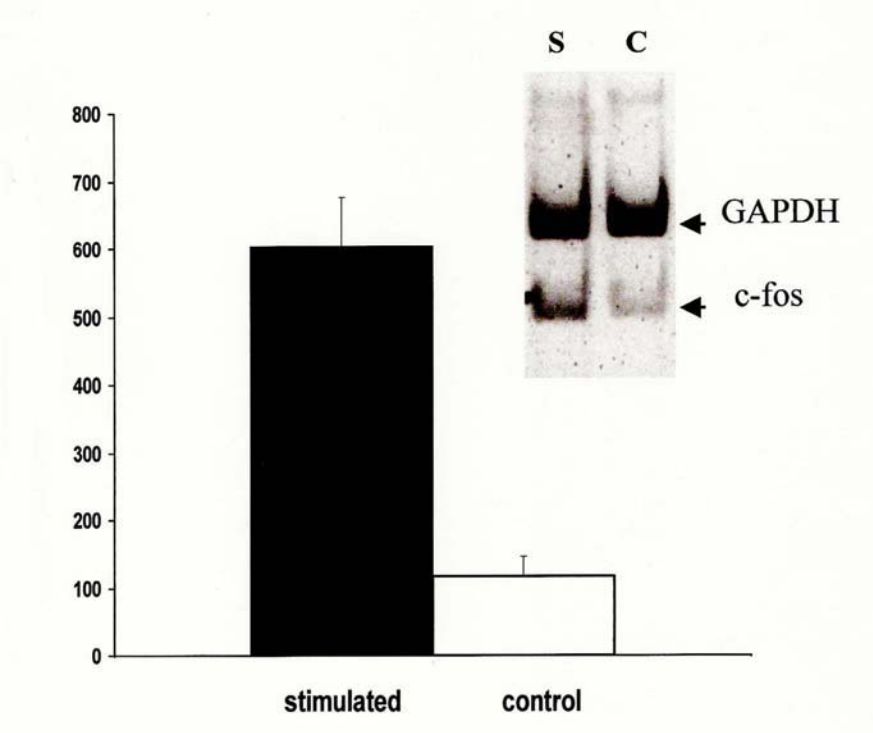


The 3 h value differs significantly from the others. SEM and the number of measurements are indicated ($p = 0.023$ between 1 and 3 h; $p = 0.004$ between 3 and 5 h). (ANOVA, *post hoc* Bonferroni test)

4.3. Expression of c-*fos* mRNA

The message of c-*fos* was detectable in the brains of control (saline-treated) and 4-AP treated animals, as was the internal control GAPDH mRNA. In the control and in the 4-AP-treated rats, the GAPDH message was not different. However, 4-AP treatment increased the level of c-*fos* mRNA highly significant, from 117 ± 28 (mean ± SEM) arbitrary units (saline-treated) to 602 ± 74 units (4-AP treated) (Figure 7).

Figure 7. The level of c-*fos* mRNA in the cerebral neocortex following 4-AP seizures (S, stimulated) and controls (C, control).



The columns are the means from three experiments, the vertical bars indicating the SEM. The difference is significant ($p < 0.05$). The inset shows the representative gel of the multiple amplification.

4.4. Changes in Fos expression following pretreatment with NMDA receptor antagonists

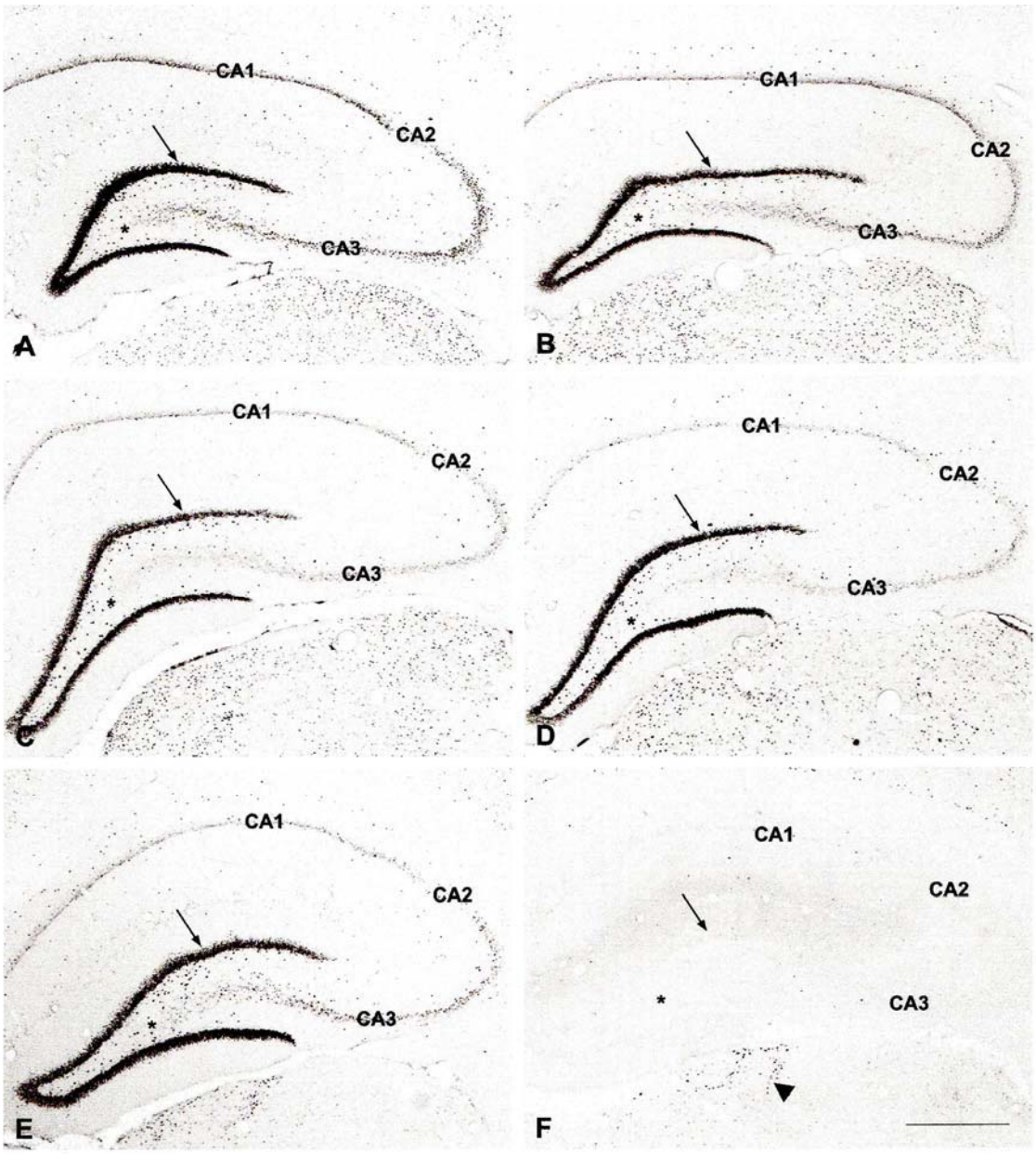
As our previous studies indicated, strong Fos immunostaining was detected in the hippocampus 3 h following 4-AP administration. Regions CA1-3 of the Ammon's horn displayed strong Fos staining, mainly in the pyramidal layer. A few scattered Fos-containing nuclei were seen in the strata oriens, radiatum and lacunosum-moleculare. The granule cell layer of the dentate gyrus displayed strong staining, whilst the hilus contained strongly stained, scattered nuclei (Figure 8A).

Pretreatment with 40 mg/kg amantadine, 40 mg/kg dextrometorphan, 3 mg/kg ketamine, or 1 mg/kg MK-801 prior to administration of 4-AP resulted in a significantly lower number of Fos-immunoreactive nuclei in CA1, CA2 and CA3 sectors of the Ammon's

horn with respect to the non-pretreated animals (injected only with 4-AP) (Figure 8B-E and 9B).

When given alone, none of the antagonists did induce a significant increase of Fos expression in the hippocampus (Figure 8F)

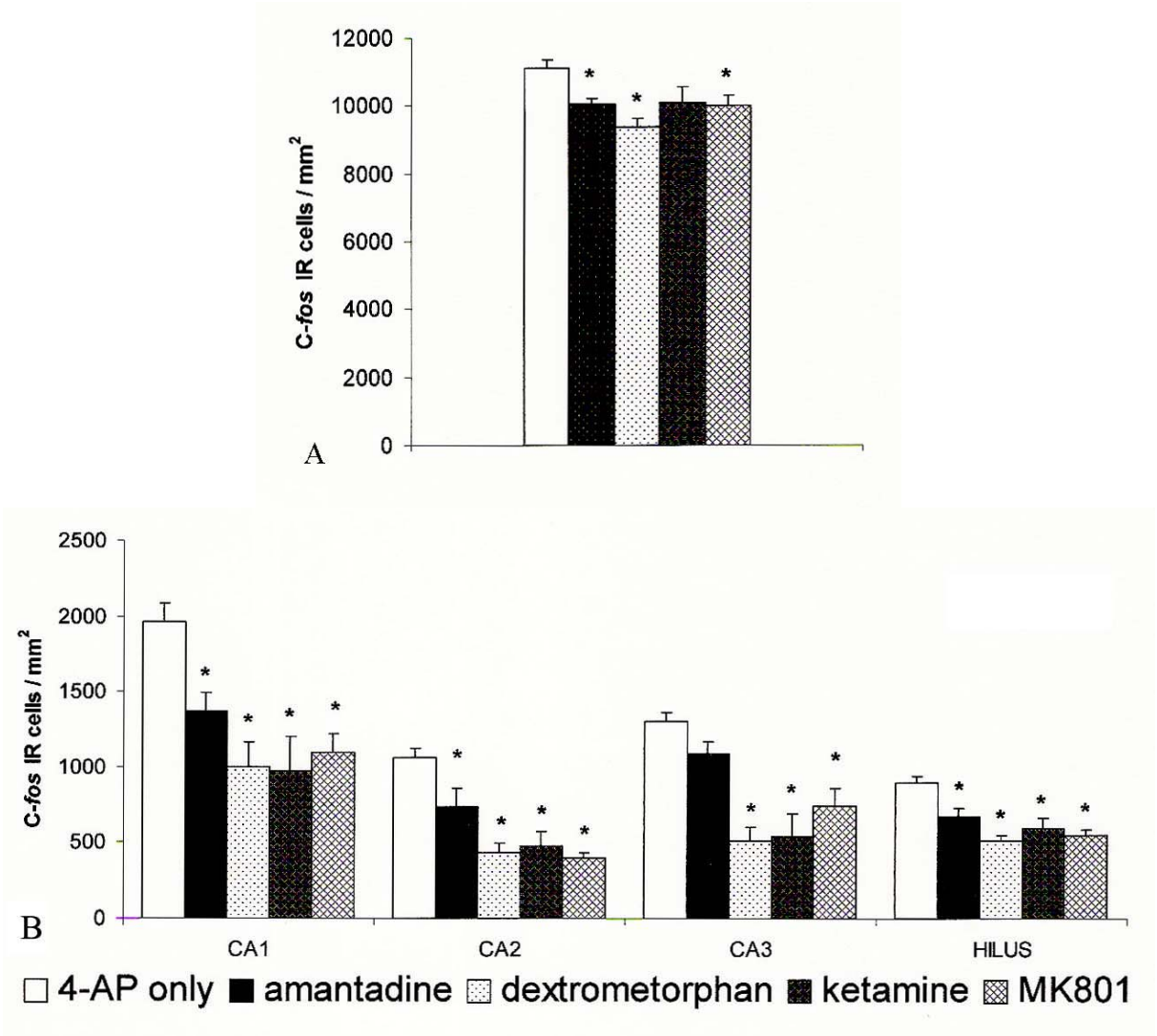
Figure 8. Low-magnification images of the distribution of Fos-positive cell nuclei in the hippocampus.



Arrow points to the granule cell layer of the dentate gyrus, while the asterisk shows the hilus of the dentate gyrus. (A) treated only with 4-AP; (B) pretreated with amantadine; (C) pretreated with dextrometorphan; (D) pretreated with ketamine; (E) pretreated with MK-801; (F) treated only with MK-801. The arrowhead in F indicates Fos staining in the dorsal thalamus. Bar: 500 μm.

The less effective among the NMDA antagonists was amantadine, which, however resulted in significant decrease in all the above regions, except for CA3 (Figure 9B).

Figure 9. Quantitative evaluation of Fos-positive cells following pretreatment with NMDA antagonists.



(A) Results of cell counts in the granule cell layer of the dentate gyrus in amantadine-, dextrometorphan-, ketamine- or MK-801-pretreated and 4-AP-injected rats compared with animals injected only with 4-AP. (B) Results of cell counts in regions CA1-3 of the hippocampus and the hilus of the dentate gyrus in antagonist-pretreated and AP-injected rats compared with animals injected only with 4-AP. Asterisks denote significant differences ($p < 0.001$; ANOVA, *post hoc* Bonferroni test); SEM is indicated. (IR: immunoreactive).

Pretreatment with amantadine, dextrometorphan, ketamine or MK-801 reduced Fos immunoreactivity in the dentate granule cell layer, although this decrease was not significant in animals pretreated with amantadine, as confirmed by statistical evaluation (Figure 9A). Moreover, pretreatment with either NMDA antagonist resulted in a significant decrease of the number of Fos-containing cell nuclei in the dentate hilus (Figure 9B).

5. DISCUSSION

5.1. Fos expression as marker of neuronal hyperactivity

Our results concerning the appearance of c-*fos* in the convulsing brain are in accord with literature data (Willoughby et al., 1995; Dragunow et al., 1987, 1989; Morgan & Curran 1991). The appearance of synchronized population spikes proved to correlate well with c-*fos* mRNA expression, which also correlated with presynaptic glutamate release. Therefore, the detection and evaluation of Fos immunostaining can be suitable for the histological mapping of neuronal hyperactivity (Labiner et al., 1993; Mihály et al., 1997, 1998). Literature data lead us to assume that 4-AP induces c-*fos* expression in part through increased release of glutamate from cerebrocortical synapses *in vivo*, and in part through the concomitantly increased Ca^{2+} influx into the postsynaptic cell. It is considered that limbic seizures produced by 4-AP are related to the enhancement of glutamatergic transmission. Recent studies proved that 4-AP increases the extracellular glutamate concentration in the rat hippocampus (Peña & Tapia, 2000). However, it should be reminded that 4-AP also releases transmitters other than glutamate: the extracellular GABA (Peña & Tapia, 2000), noradrenaline (Versteeg et al., 1995) and dopamine (Bonano et al., 2000) concentrations increased following 4-AP treatment both *in vivo* and *in vitro*.

Extensive literature evidence supports the role of different glutamate receptors in c-*fos* gene expression. Antagonists of the NMDA receptor inhibited seizure-induced c-*fos* mRNA expression in the granule cells of the dentate gyrus, suggesting that dentate neuronal hyperactivity marked by c-*fos* induction is mediated by NMDA receptor activation (Labiner et al., 1993). Accordingly, decreased seizure-induced c-*fos* expression following NMDA antagonist drugs indicates the attenuated postsynaptic effects of glutamate and concomitant influx of Ca^{2+} (Greenberg & Ziff, 2001). Furthermore, immunohistochemical studies with NMDA receptor subunit NR1 antibody revealed strong staining in the molecular layer and

hilus of the dentate gyrus, as well as in sectors CA1 and CA3 of the Ammon's horn (Johnson et al., 1996), which are innervated by glutamate synapses. However, not only NMDA, but also AMPA and kainate receptors are localized on the postsynaptic membranes of hippocampal neurons (Löscher, 1998), and both NMDA and non-NMDA receptors are involved in the epileptiform discharges mediated by the perforant pathway in the dentate gyrus (Jones & Lambert, 1990). Extracellular glutamate acts on these ionotropic receptors: AMPA receptors trigger depolarization and neuronal burst initiation leading to the activation of NMDA receptors and the opening of the ion channel generates Ca^{2+} influx (Hwa & Avoli, 1991; Löscher 1998). The blockade of the NMDA channel by non-competitive NMDA antagonists inhibits or delays the influx of Ca^{2+}, which in turn should delay or inhibit the long-lasting neuronal depolarization (Hwa & Avoli, 1991). Since c-*fos* induction (through membrane depolarization and Ca^{2+} influx) is a graded response once it reaches a minimal threshold for expression (Labiner et al., 1993), different c-*fos* expression patterns (resulting in a variation in the number of Fos-immunoreactive cells and different staining intensity) is plausible. However, our studies indicated that in the 4-AP model strong and reproductive Fos immunostaining was observed in the hippocampus (as well as in the neocortex) at 3 h following the i.p. administration of 4-AP. Our investigations revealed that this time of exposure to 4-AP is within the interval in which Fos protein can be identified in the dentate granule cells, and is maximal in the CA1-3 sectors of the Ammon's horn. We, therefore, believe that careful counting of the Fos-positive cell nuclei at this interval serves as an indicator of neuronal hyperactivity in forebrain structures *in vivo*, and thereby the 4-AP model is a reliable one for the pharmacological investigation of the synaptic mechanisms of the hippocampus.

5.2. Neuroanatomical aspects of hippocampal seizures

Our experiments indicated that a very strong activation of the dentate gyrus occurs at 1 h following intraperitoneal 4-AP administration. This finding is similar to others in the literature relating to other convulsants, although there are some differences in time, depending on the nature of the chemical used (Herdegen & Leah, 1998). Literature data lead us to consider that experimental limbic seizures start from the entorhinal cortex both *in vivo* (Collins et al., 1983) and *in vitro* (Jones, 1993; Barbarosie et al., 2000). Distinct cell

populations of the rodent entorhinal cortex project to the dentate gyrus and to the CA1 and CA3 sectors of the Ammon's horn in the perforant pathway, which is thought to be glutamatergic. This pathway activates the dentate granule cells and the pyramidal neurons of regions CA1 and CA3 (Jones, 1993). It also terminates on inhibitory interneurons of regions CA1 (Gulyás et al., 1999) and CA3 (Freund & Buzsáki, 1996). This suggests that entorhinal afferents to regions CA1-3 are divergent and probably less effective than those ending in the dentate gyrus. Furthermore, when activated by the perforant pathway, the dentate granule cells may prevent the spread of activity towards the Ammon's horn: the mossy fibers innervate not only the CA3 pyramidal cells, but also inhibitory GABAergic interneurons of the hilus and region CA3, which probably exert a feed-forward inhibition on the CA3 pyramidal cells, delaying activation of the Schaffer collateral system (Acsády et al., 1998). This complex neuroanatomical stucture and synaptic connections may explain our findings concerning the differences in the numbers and staining intensities of Fos-immunoreactive cells between the dentate gyrus and Ammon's horn. Increased staining intensity in the Ammon's horn with a staining intensity decrease in the dentate granule layer at 3 h probably reflects the overcoming by excitation of the inhibitory influences in regions CA1 and CA3 (including the mossy fiber-driven inhibition). The appearance of Fos immunoreactivity in the hilus of the dentate gyrus followed that of the granule cell layer: the numbers of Fos-positive cell nuclei in these areas were highest at 1 h, and subsequently gradually decreased. This can be explained on the basis of the proximity of the hilar neurons and granule cells: large variety of hilar neurons (Amaral, 1978) receive input from the mossy fibers (Frotscher et al., 1991), this input being convergent and very effective (Acsády et al., 1998).

5.3. Fos expression in PV-containing interneurons in the hippocampus

The selective occurrence of the Ca^{2+}-binding protein parvalbumin (PV) in GABAergic interneurons was confirmed in the cerebral cortex (Celio, 1986), as well as in the hippocampus (Kosaka et al., 1987). Hippocampal PV-containing neurons comprise a characteristic population of GABAergic neurons: based on their morphology and laminar distribution in the Ammon's horn, they include basket cells and axo-axonic cells, which mediate perisomatic inhibition (Kosaka et al., 1987; Gulyás et al., 1999). A similar perisomatic inhibition occurs in the dentate granule cell layer (Freund & Buzsáki, 1986).

Immunohistochemical detection of PV-containing neurons reveals that PV-immunoreactive cell bodies are located in the stratum pyramidale and stratum oriens of the CA1 and CA3 subfields of the hippocampus and in the granule cell layer and hilus of the dentate gyrus. PV-GABA colocalization studies indicate that PV-containing cells are also glutamic acid decarboxylase (synthesizing enzyme of GABA, GAD) immunoreactive, whereas the overall proportion of GAD-positive neurons containing PV is about 20% in the hippocampus (Kosaka et al., 1987). Nevertheless, because the majority of PV-positive cell bodies are within or adjacent to the principal cell layer, they account for approximately half of the GABAergic neurons in these areas (Freund & Buzsáki, 1986). An interesting observation in several colocalization studies is the weak GABA immunoreactivity of PV-positive somata compared to other GABAergic cell bodies lacking this Ca^{2+}-binding protein (Kosaka et al., 1987; Gulyás et al., 1991; Aika et al., 1994). This might be related to high spontaneous firing and extensive transmitter release, which requires a rapid transport of the synthesizing enzyme to the terminals and consequently allows little transport of GABA back to the soma (Freund & Buzsáki, 1986). Moreover, recent immunohistochemical studies have revealed that a subpopulation of PV-containing interneurons in the hippocampus have an AMPA receptor profile which is likely to make these interneurons selectively vulnerable to excitotoxicity (Moga et al., 2002). As mentions above, AMPA receptors are important mediators of excitatory neurotransmission; they consist of variable combinations of four subunits (GluR1-4) of which GluR1, GluR3 and Glu4 form channels with high Ca^{2+} permeabilities, while GluR2 inhibits Ca^{2+} influx through AMPA channels when present in the receptor complex (Hollmann & Heinemann, 1994). The expression pattern of AMPA receptor subunits in a subset of PV-containing interneurons (intense GluR3 immunoreactivity and lack of GluR2 subunit) would result in high Ca^{2+} permeability (Moga et al., 2002), and these neurons are likely to be selectively vulnerable to excitotoxicity. These PV-containing GABAergic interneurons may play a critical role in seizure process (Moga et al., 2002) as decrease in inhibitory control is an important mechanism for seizure genesis (Olsen & Avoli, 1997). Accordingly, it has been demonstrated that hilar GABAergic cells are vulnerable to seizure-induced damage (while dentate basket cells are resistant; Avoli et al., 2002).

PV-containing neurons in the hilus can exert a feed-back inhibition on the granule cells, because they are contacted by mossy fibers (Nitsch et al., 1990). Our findings indicated that the number of activated PV neurons was highest at 3 h not only in the Ammon's horn, but also in the dentate region. This feature of PV neuron activation in region CA1 is not

surprising, because the afferents of the pyramidal cells and those of the PV cells are similar (Gulyás et al., 1999). The synaptic connections are more complex in region CA3: part of the input to the PV-containing interneurons comes through the mossy fibers from the granule cells (Dragunow et al., 1992), which proved to be activated earlier than the cells of region CA3 in our experiments. The highest number of the activated PV cells in the dentate region was found at 3 h, indicating that Fos expression is not uniform in the hilar neurons: some hilar cells exhibit early c-*fos* gene activation, whereas some (PV-containing) cells are activated later. However, it should be mentioned that the number of PV cells in the hilus is relatively small (Nitsch et al., 1990). This may explain the early peaking of the Fos-positive cell counts in the hilus, and the discrepancy between the activation of the PV-containing neurons and the activation of the total hilar cell population. It was, however, a characteristic feature of the dentate PV cell activation that it outlasted the Fos expression of the granule cells. This observation is similar to others in the literature related to somatostatin-containing cells in the dentate hilus: Fos staining in somatostatin interneurons outlasted that of the granule cells (Dragunow et al., 1992). Interestingly, PV-containing interneurons in the rat hippocampus express protein subunits of the delayed rectifier potassium channel, and the PV neurons are sensitive to low 4-AP concentrations, resulting in the increased amplitude and duration of the action potential (Du et al., 1996). This indicates that dentate PV cells could have been directly affected by 4-AP, which could contribute to their Fos expression pattern. This long-lasting Fos expression of the PV-containing interneurons of the dentate hilus suggests some long-lasting cellular alterations, or eventual future cell death. It has been shown that long lasting seizures induced heat shock protein expression in vulnerable hilar neurons, as an indicator of excitation-induced cellular injury (Sloviter & Lowenstein, 1992). In our experiments, it seems that limbic seizures first increase the number of PV neurons, and when the seizure activity has disappeared, the number of PV cells decreases again. However, this issue must be proved by means of in-situ hybridization or Northern blotting of specific mRNA.

5.4. Fos expression in the hippocampus and NMDA antagonist drugs

As discussed above, the 4-AP model proved to be reliable for the pharmacological assessment of seizure genesis and spread in forebrain structures *in vivo*, and quantitation of the Fos protein-immunostained cell nuclei serves as an indicator of neuronal hyperactivity.

Previous studies from our laboratory indicated that the Fos protein was detectable in the forebrain by Western blotting in 4-AP seizures (Mihály et al., 1997). In the present experiments, the RT-PCR studies demonstrated the induction of c-*fos* mRNA in the cerebral cortex following 4-AP administration.

Literature evidences lead us to assume that neuronal hyperactivity and thereby Fos induction produced by 4-AP are related to the enhancement of glutamatergic transmission. On the basis of our evaluation we conclude that all the examined NMDA receptor antagonist drugs decreased significantly the seizure-induced expression of c-*fos* in the hippocampus. It is, however, important to mention that only ketamine, MK-801 and dextrometorphan caused symptomatic seizure protection: ketamine and dextrometorphan increased the latency of the GTCS significantly; MK-801, ketamine and dextrometorphan decreased the incidence of the GTCS. Amantadine did not attenuate the symptoms of the seizure. We, therefore, consider that the immunohistochemical results reflect the antagonistic effect of the drugs on the receptor level, and they can be correlated only indirectly with the behavioural effects shown by the animals.

The non-competitive NMDA antagonist ketamine, MK-801, amantadine and dextrometorphan decrease the postsynaptic effects of glutamate mainly by blocking the NMDA receptor channel. Isotope-labelled derivates of some of these compounds are used in human brain imaging of the glutamatergic system *in vivo* in hope of gaining insight into the pathophysiology of schizophrenia (Bressan & Pilowsky, 2000). It should be reminded, in this respect, that a variety of NMDA receptor antagonist drugs such as ketamine can mimic, more faithfully than other psychotomimetics (e.g. amphetamines, LSD), a broad range of symptoms seen in schizophrenia, including impairments of cognitive function (Malhotra et al., 1996; Neycomer, 1999). Additionally, administering an NMDA antagonist drug (e. g. MK-801) in high dosage or by continuous infusion for several days induces a prolonged NMDA receptor *hypo*functional state which causes irreversible neuronal injury in cerebrocortical and limbic regions, including entorhinal and prefrontal cortices, and the hippocampus (Ellison, 1994; Horvath et al., 1997). These neurotoxic and psychotic effects of NMDA antagonist drugs arise from the *hypo*activation of NMDA receptors (Farber et al., 1998).

There are multiple recognition sites associated with the NMDA receptor through which receptor channel function is modulated. Ketamine and MK-801 are high-affinity open-channel blockers acting at the phencyclidine (PCP) site and thereby they inhibit or decrease the ion fluxes which follow the glutamate binding (Farber et al., 1998). Extensive research

proved the central role of NMDA receptor hyperfunction in the pathophysiology of epileptiform activity (Olney, 1990). Intraperitoneal or intracerebroventricular administration of the NMDA antagonist MK-801 was found to protect against 4-AP seizures in electrophysiological studies (Morales-Villagrán et al., 1996). Our experiments provided further data on seizure protection and indicated that pretreatment by NMDA antagonists prevent GTCS (except for amantadine) and reduces c-*fos* induction in the hippocampus.

Literature data on the effects of ketamine are not unequivocal: no effects were seen on the epileptiform activity induced by 4-AP in hippocampal slices (Sagratella, 1987), but ketamine was found to be effective against picrotoxin seizures *in vitro* (Lee & Hablitz, 1990) and electroconvulsions *in vivo* (Stewart & Reid, 1994). Ketamine has been reported to be useful in the therapy of refractory status epilepticus in humans (Sheth, 1998). Ketamine and its isomers were found to possess neuroprotective effects in seizure-related brain damage (Clifford et al., 1989) and in global forebrain ischaemia (Proescholdt et al., 2001). In the present experiments, increased GTCS latency data were indicative of an anticonvulsant role of ketamine in 4-AP seizures. The decrease in c-*fos* expression is clearly a sign of decreased Ca^{2+} influx into the neurons. These effects might be related to the NMDA receptor antagonism of ketamine and its blocking action on Na^{+} channels (Zhou & Zhao, 2000).

Amantadine and dextrometorphan are low-affinity open-channel blockers. Amantadine belongs to the family of aminoadamantanes, which is known to display antiparkinsonian-like activity as well as neuroprotective action (Danysz et al., 1997). Memantine (1-amino-3,5-dimethyladamantane) has better therapeutic indices than amantadine and it has already been used in the therapy of Azheimer's disease (AD). Based on evidence that a severe loss of NMDA receptor-bearing neurons is present in the human AD brain (Olney et al., 1998), which might arise from the amyloidosis-induced increased sensitivity of NMDA receptors to even normal concentrations of glutamate (Farber et al., 1998), the NMDA antagonist memantine proved to be effective in AD. Memantine was found to decrease tonic activation of NMDA receptors (noise), and thereby improves neuronal plasticity and cognition (Parsons et al., 1999). The present experiments indicate that amantadine decreases seizure-induced c-*fos* expression in the hippocampus, with the only exception of the CA3 region. Possibly, Ca^{2+} fluxes are able to displace the antagonist from the channel resulting in the cessation of its effect. We, therefore, conclude that amantadine probably decreases transiently the Ca^{2+} influx through the NMDA receptor, and this was reflected by the decrease of seizure-induced c-*fos* expression. However, amantadine does not provide symptomatic seizure protection.

The effects of dextrometorphan are more complex. This compound inhibits NMDA-induced convulsions and is, therefore, regarded as a non-competitive antagonist (Cole et al., 1989, Ferkany et al., 1988). It also decreases kainic acid seizures in rat and attenuates the consequent hippocampal neuronal damage (Kim et al., 1996). Moreover, it inhibits ischaemia-induced c-*fos* expression and neuronal death in hippocampal neurons (Bokesch et al., 1994). Some of the effects of dextrometorphan are mediated by the NMDA receptors, and some by the voltage-dependent Ca^{2+} and Na^{+} channels (Trube & Netzer, 1994). The effects of dextrometorphan observed in our experiments are promising: the overall decrease of seizure-elicited Fos immunoreactivity in CA1-3 regions of the hippocampus indicated that the Ca^{2+} influx was substantially inhibited. Furthermore, dextrometorphan increased significantly the latency of the GTCS.

It is, however, important to emphasize that GTCS, which represents the main symptoms of the 4-AP treatment, did not show a strict correlation with c-*fos* expression. Animals pretreated with NMDA antagonists and exhibiting decreased c-*fos* expression displayed GTCS, although the latency and incidence of the symptoms were affected significantly. One explanation of this observation may be that the induction of the c-*fos* gene in response to increased release of glutamate occurs in a critical period, during which increase of glutamate release and facilitation of voltage-dependent Ca^{2+} channels trigger those intracellular cascades which lead to c-*fos* expression (Labiner et al, 1993; Greenberg & Ziff, 2001). Blockade of the NMDA receptor channel during this period could inhibit the induction of the c-*fos* gene, whereas glutamate release after this critical period may not induce further c-*fos* expression, but may cause and maintain the symptoms. This could explain the discrepancy between the occurrence of GTCS and the decrease of Fos immunoreactivity. It is supposed, therefore, that the immunohistochemical results reflect the antagonistic effects of the NMDA blockers on receptor level, and could not be strictly correlated with symptomatic seizure protection. Another explanation could be related to the role of non-NMDA receptors in the development and maintenance of the symptoms, as supported by literature data (Chapman, 1998; Löscher, 1998). Both NMDA and non-NMDA receptors play a role in the epileptiform discharges mediated by the perforant path in the dentate gyrus (Jones & Lambert, 1990). Immunohistochemical studies have confirmed the presence of AMPA subunits in the molecular layer of the dentate gyrus (Molnár et al., 1993). These observations explain our finding that the non-competitive NMDA antagonists exert a similar effect in all regions of the hippocampal formation, in which they attenuate, but do not abolish Fos protein

immunostaining. Although the overall decrease in Fos immunoreactivity in the hippocampus after pretreatment with NMDA antagonists indicated the importance of NMDA-mediated glutamate action in the genesis and maintenance of the neuronal hyperactivity, the remaining Fos immunostaining revealed the importance of non-NMDA glutamate receptors, and probably other transmitter system, in the synaptic mechanisms of the hippocampus.

6. CONCLUSIONS

In our experiments we established a seizure model reliable for the pharmacological investigation of the synaptic mechanisms of the hippocampus.

1. Evaluation and careful counting of the Fos-immunoreactive cell nuclei serves as an indicator of neuronal hyperactivity in the hippocampus *in vivo*.
2. Strong Fos immunostaining was observed in the hippocampus at 3h following the i.p. administration of 4-AP, therefore this time of exposure to the convulsant agent is suitable for immunohistochemistry and quantitation.
3. Activation of the parvalbumin-containing interneurons in the dentate hilus outlasted Fos expression of the granule cells, indicating some long-lasting cellular alterations in hilar PV neurons.
4. All the examined NMDA receptor antagonist drugs decreased significantly the seizure-induced c-*fos* expression in the hippocampus, revealing the involvement of NMDA receptors in this process.
5. This 4-AP model could be useful for further pharmacological assessments of the glutamatergic neurotransmission of the hippocampus, especially for the investigation of NMDA receptors, which are thought to play an important role in the pathomechanism of major psychiatric disorders.

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9. APPENDIX: Papers related to the thesis