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

Summary of Ph.D.Thesis

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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
AOI	area of interest
4-AP	4-aminopyridine
CA	cornu Ammonis
cAMP	3',5'-cyclic adenosine monophosphate
DAB	diaminobenzidine tetrahydrochloride
GABA	gamma aminobutyric acid
GAD	glutamic acid decarboxylase
GADPH	glyceraldehyde 3-phosphate dehydrogenase
GluR	glutamate receptor
GTCS	generalized tonic-clonic seizures
IEG	immediate early gene
IgG	immunoglobulin G
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
PCP	phencyclidine
PCR	polymerase chain reaction
PV	parvalbumin

1. INTRODUCTION

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. The **c-*fos* protooncogene** belongs to the inducible transcription factors, 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. 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. 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. Seizure activity induced by various types of convulsant agents leads to a rapid, massive and transient appearance of Fos protein throughout those brain regions that normally express low basal levels. The postsynaptic c-*fos* mRNA expression correlates well with the presynaptic release of excitatory neurotransmitters, therefore the immunohistochemical detection and evaluation of Fos protein appears suitable for the histological mapping of neuronal hyperactivity.

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. *In vitro* studies on the involvement of excitatory amino acid receptors have demonstrated that glutamate receptors of the NMDA and AMPA types, as well as the nicotinic acetylcholine receptor are able to activate c-*fos* expression. *In vivo*, the NMDA and AMPA receptors are the main candidates for c-*fos* induction.

The compound **4-aminopyridine (4-AP)** is a blocker of K^+ conductances. The delay in neuronal repolarisation increases transmitter release and augments inhibitory and excitatory postsynaptic potentials. Focal and systemic administration of 4-AP induces rapidly an intense expression of c-*fos* in the neocortex and hippocampus. 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. 4-AP acts on several hippocampal

pathways and augments the release of glutamate from their synapses. It is considered, 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**. Blockade of the NMDA receptors inhibits the expression of c-*fos* mRNA in the dentate granule cells, suggesting that neuronal hyperactivity and thereby c-*fos* induction is 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+}.

Glutamate causes a marked increase in the firing rate of granule and pyramidal cells and is the major excitatory transmitter in the hippocampus. The main glutamatergic afferents to the hippocampus are represented by the perforanth path originating in the entorhinal cortex and terminating 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. CA3 pyramidal cells also receive a direct projection from the entorhinal cortex. It is, however, important to emphasize that mossy fiber collaterals form synapses in the hilus with a great number of inhibitory interneurons, and also provide innervation to excitatory, glutamatergic hilar mossy cells. The inhibitory hilar interneurons provide GABAergic input to CA3 pyramidal cells and thus lead to feedforward inhibition following mossy fiber activity. CA1 area is the major target of CA3 pyramidal cell axons, the Schaffer collaterals, and the pyramidal cells of this subfield represent the 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. **Glutamate,** as **the major excitatory neurotransmitter in the hippocampus**, is involved in long-term potentiation (LTP), synaptic plasticity, epileptic seizures, excitotoxicity and neurodegeneration. Both LTP and LTD (long-term depression) 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. In particular, glutamate and **NMDA receptors** play an important role in hippocampal functional changes and structural plasticity, including synapse formation in CA1

region, regulation of adult neurogenesis in the dentate granule cells or remodeling of apical dendrites in CA3 area.

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 receptor family is the most frequently implicated in functional and structural changes seen in several neuropsychiatric disorders. 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. 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. 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. 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. 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 PTSD.

Glutamate and NMDA receptors are also involved in neuronal death in pyramidal cells of the hippocampus following seizures and ischaemia. 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. The competitive and selective NMDA antagonists have been shown to be potent antiepileptic agents and are particularly effective against reflex epilepsy and chemically-induced seizures. High-affinity open-channel NMDA receptor blockers such as PCP, ketamine and dizocilpine (MK-801) are also potent anticonvulsants, and protect against seizure-related brain damage. Low-affinity open-channel NMDA receptor blockers such as amantadine, memantine, remacemide, dextrometorphan and its metabolite dextrorphan also display anticonvulsant and neuroprotective activities. Some of these NMDA antagonist drugs are already in clinical use.

2. OBJECTIVES

The aims of our studies were to

i.) 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.) 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.) 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 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 (180-200 g). The i.p. administration of 4-AP (5 mg/kg b.w.) caused symptoms of GTCS 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, the animals were anaesthetized, the brains were removed and processed for immunohistochemistry.

3.2. C-*fos* immunohistochemistry

Polyclonal c-*fos* antibody and the PAP method were used. Primary c-*fos* antibody was followed by donkey anti-rabbit IgG; the secondary antibody was detected by the PAP technique and the peroxidase reaction was localized with Ni-DAB.

3.3. Parvalbumin (PV) and Fos double immunostaining

Primary antibody cocktails (mouse anti-PV and rabbit anti-c-*fos*) were used. Primary antibodies were followed by biotinylated anti-mouse IgG and plain donkey-anti-rabbit IgG, detected by streptavidin-peroxidase and PAP, respectively. The streptavidin-peroxidase was developed by using DAB.

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

Pretreatment 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.) was performed in four groups. Animals were injected i.p., 10 min prior to the application of 4-AP (5 mg/kg b.w.). One control group received the same amount of solvent and 4-AP (5 mg/kg b.w.). Other control groups received only the tested drugs, without 4-AP. Finally, an additional control group received only physiological saline. Three hours after the administration of 4-AP, the animals were anaesthetized and the brains were processed for immunohistochemistry. The latency of the onset of GTCS from the time of 4-AP injection, was evaluated in parallel experiments, in groups of 15 animals each (75 animals in total).

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. 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. 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. 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. 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.

On double-stained histological sections, the neurons containing *Fos plus PV* or *PV only* were counted separately, 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.

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 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. We analysed the Fos-containing cell nuclei in the hippocampus 1, 3, and 5 h following the injection of 4-AP. 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. 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. Some scattered Fos-positive cell nuclei were seen in the molecular layer of the dentate gyrus, whilst the hilus contained many more activated cells. 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.

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 CA1-3, and a 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. Regions CA1-3 of the hippocampus displayed strong Fos-like staining, mainly in the pyramidal layer. 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. 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.

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. Apart from the hilus, a few scattered cells were found in the granule cell layer and in the molecular layer. The number of PV-positive neurons expressing Fos protein (PV-Fos neurons) in the Ammon's horn and in the dentate gyrus increased between 1 and 3 h, and then was strongly reduced by 5 h post-injection. 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.

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.

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 arbitrary units (saline-treated) to 602 ± 74 units (4-AP treated).

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

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). When given alone, none of the antagonists did induce a significant increase of Fos expression in the hippocampus 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. Moreover, pretreatment with either NMDA antagonist resulted in a significant decrease of the number of Fos-containing cell nuclei in the dentate hilus.

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. 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. 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. However, it should be reminded that 4-AP also releases transmitters other than glutamate (e.g. GABA, noradrenalin

and dopamine). 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. Accordingly, decreased seizure-induced c-*fos* expression following NMDA antagonist drugs indicates the attenuated postsynaptic effects of glutamate and concomitant influx of Ca^{2+}. 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. Since c-*fos* induction is a graded response once it reaches a minimal threshold for expression, different c-*fos* expression patterns (resulting in a variation in the number of Fos-immunoreactive cells and different staining intensity) are plausible. However, our studies indicated that in the 4-AP model strong and reproductive Fos immunostaining was observed in the hippocampus 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 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. Literature data lead us to consider that experimental limbic seizures start from the entorhinal cortex both *in vivo* and *in vitro*. Distinct cell populations of the rodent entorhinal cortex project to the dentate gyrus and to the CA1-3 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 CA1-3 and t also terminates on inhibitory interneurons of regions CA1-CA3. 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 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. 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 receive input from the mossy fibers, this input being convergent and very effective.

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

The selective occurrence of the Ca^{2+}-binding protein PV in GABAergic interneurons was confirmed in the cerebral cortex, as well as in the hippocampus. Hippocampal PV-containing neurons comprise a characteristic population of GABAergic neurons: they include basket cells and axo-axonic cells, which mediate perisomatic inhibition. A similar perisomatic inhibition occurs in the dentate granule cell layer. PV-GABA colocalization studies indicate that PV-containing cells are also GAD immunoreactive, whereas the overall proportion of GAD-positive neurons containing PV is about 20% in the hippocampus. 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. 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. 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, and these neurons are likely to be selectively vulnerable to excitotoxicity. These PV-containing GABAergic interneurons may play a

critical role in seizure process as decrease in inhibitory control is an important mechanism for seizure genesis. Accordingly, it has been demonstrated that hilar GABAergic cells are vulnerable to seizure-induced damage (while dentate basket cells are resistant). PV-containing neurons in the hilus can exert a feed-back inhibition on the granule cells, because they are contacted by mossy fibers. 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. 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, 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. 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. 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. 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.

5.4. Fos expression in the hippocampus and NMDA antagonist drugs

In our experiments all the examined NMDA receptor antagonist drugs decreased significantly the seizure-induced expression of c-*fos* in the hippocampus. The non-competitive NMDA antagonist ketamine, MK-801, amantadine and dextrometorphan decrease the postsynaptic effects of glutamate mainly by blocking the NMDA receptor

channel. 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. However, 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, but ketamine was found to be effective against picrotoxin seizures *in vitro* and electroconvulsions *in vivo*. Ketamine has been reported to be useful in the therapy of refractory status epilepticus and to possess neuroprotective effects in seizure-related brain damage and in global forebrain ischaemia. 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.

Amantadine is known to display antiparkinsonian-like activity as well as neuroprotective action. 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. It has been reported to decrease kainic acid seizures and to attenuate consequent hippocampal neuronal damage. Moreover, it inhibits ischaemia-induced c-*fos* expression and neuronal death in hippocampal neurons. Some of the effects of dextrometorphan are mediated by the NMDA receptors, and some by the voltage-dependent Ca^{2+} and Na^{+} channels. 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. 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. 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.

i. Evaluation and careful counting of the Fos-immunoreactive cell nuclei serves as an indicator of neuronal hyperactivity in the hippocampus *in vivo*.
ii. 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.
iii. Activation of the hilar PV-containing interneurons outlasted Fos expression of the granule cells, indicating some long-lasting cellular alterations.

iv. 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.

v. 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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