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Non-competitive NMDA receptor antagonists moderate seizure-induced c-*fos* expression in the rat cerebral cortex

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ABSTRACT: We examined the effects of non-competitive NMDA glutamate receptor antagonists on seizures elicited by 4-aminopyridine (4-AP), and in particular, on the expression of the transcription factor c-*fos* induced by these seizures. Induction of c-*fos* mRNA due to 4-AP-elicited seizures was ascertained by reverse transcription polymerase chain reaction in samples of the neocortex. Adult rats were pretreated with the NMDA receptor antagonists amantadine (40 mg/kg), ketamine (3 mg/kg), dizocilpine (MK-801; 1 mg/kg) or dextrometorphan (40 mg/kg); 4-AP (5 mg/kg) was then injected i.p. Controls were treated with either antagonist only or with 4-AP only. Pretreatment with the antagonists (with the exception of amantadine) increased the latency of behavioural seizures, but not all of the antagonists caused symptomatic seizure protection. In the brains which were processed for Fos immunohistochemistry, quantitative evaluation of immunostained cells was performed in the neocortex and hippocampus. Treatment with either antagonist did not induce by itself c-*fos* expression, with the exception of amantadine, which caused slight Fos induction in the neocortex. Pretreatment with all the antagonists resulted in decrease of seizure-induced Fos immunoreactivity with respect to non-pretreated animals. Decrease of immunostained cells was significant in the neocortex, in the granule cell layer and hilus of the dentate gyrus, in hippocampal areas CA1 and CA2. MK-801, ketamine and dextrometorphan decreased significantly Fos immunoreactivity also in area CA3. The decrease of Fos immunostaining was not directly correlated with a suppression of behavioural seizures. The results support an important role of NMDA receptors in c-*fos* gene induction in acute 4-AP seizures.
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KEY WORDS: Epilepsy, 4-Aminopyridine, Gene expression, Immunohistochemistry, Glutamate receptor, Rat.

INTRODUCTION

The c-*fos* protooncogene is an inducible transcription factor [22], which belongs to the AP-1 family and exerts various regulatory actions in the cell nucleus. Very little is known about the genes which are regulated through the action of c-*fos*, but the signals and intracellular pathways which lead to expression of the c-*fos* gene have been identified. In general, membrane depolarisation, Ca^{2+} influx, cAMP and some growth factors are able to influence c-*fos* expression, predominantly by means of intracellular protein kinase cascades. Expression of c-*fos*, typically induced by epileptic seizures, is mediated mainly by transmitters acting on ionotropic receptors and the voltage-dependent Ca^{2+} channels in the membrane [20]. *In vitro* experiments have demonstrated that the nicotinic acetylcholine receptor, and glutamate receptors of the *N*-methyl-*d*-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) types, are able to activate c-*fos* expression [22]. *In vivo*, the NMDA and AMPA receptors are the main candidates for such activation [20]. The AP-1 regulatory proteins (Fos, Fra and Jun) participate in the regulation of degeneration and regeneration in the developing and adult brain (details of this regulation have recently been discovered [21]).

The compound 4-aminopyridine (4-AP) is a blocker of K^+ conductances, and specifically those of the $I_{K(A)}$ and $I_{K(V)}$ currents [1]. The delay in neuronal repolarisation increases transmitter release and augments inhibitory and excitatory postsynaptic potentials [45,53,57]. If injected i.p., 4-AP causes generalised seizures [34]. Our previous studies indicated that focal and systemic administration of 4-AP induces rapidly an intense and long-lasting expression of c-*fos* in the neocortex and hippocampus [35,37]. Based on the mechanism of action of 4-AP, this effect was presumed 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 [18,25]. Both NMDA and non-NMDA glutamate receptors are known to contribute to the *in vitro* ictagenic effects of 4-AP [18]. Studies involving intracellular recording in rat neocortical slices have shown that NMDA receptors contribute to the process of stimulus-induced paroxysmal depolarisation shift amplification by prolonging the duration and reducing the latency of the epileptiform discharge [25].

The anticonvulsant properties of glutamate receptor antagonist drugs have been reported in various *in vivo* and *in vitro* epilepsy models [32,42,48]. The competitive and selective NMDA antagonists 2-amino-7-phosphonoheptanoic acid (AP7)

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and 2-amino-5-phosphonovaleric acid (AP5) have been demonstrated to be potent antiepileptic agents when given i.c.v. to mice [12,33]. High-affinity open-channel NMDA receptor blockers such as phencyclidine, ketamine and dizocilpine (MK-801) are also potent anticonvulsants [3,56] and protect against seizure-related brain damage [9,10]. Low-affinity open-channel NMDA receptor blockers such as amantadine (1-aminoadamantane), memantine (1-amino-3,5-dimethyladamantane), remacemide, dextrometorphan ((+)-3-methoxy-*N*-methylmorphinan) and its metabolite dextrorphan also display anticonvulsant and neuroprotective activities [24,41,47–49]. Some of these antagonists are already in clinical use [6]. The aim of the present study 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.

MATERIALS AND METHODS

Animals and Treatment

Experiments were performed on a total of 111 male Wistar rats weighing 180–200 g. The experiments were conducted in accordance with prevailing laws and ethical considerations. Written permission was obtained in advance from the Faculty Ethical Committee on Animal Experiments (University of Szeged). The animals had free access to food and water. The convulsant agent 4-AP (Sigma, St. Louis, MO) was dissolved in saline (0.67 mg in 1 ml vehicle) and administered i.p. (5 mg/kg). In previous investigations, this dose proved to be epileptogenic [34,35].

Pretreatment with ketamine (3 mg/kg), MK-801 (1 mg/kg), amantadine (40 mg/kg) or dextrometorphan (40 mg/kg) (all purchased from Sigma) was performed in four groups, each containing three animals (12 animals). The tested 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). 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 were used for immunohistochemistry (30 rats in total). At the end of the experiment, 3 h after the i.p. injections of 4-AP, the animals were deeply anaesthetised with diethyl ether and perfused transcardially with 200 ml of 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 300 ml of fixative (4% phosphate-buffered paraformaldehyde, pH 7.4). This time of exposure to 4-AP is within the interval in which the Fos protein can be identified or is maximal in the neurons that express the c-*fos* gene [17,35,59].

The brains were rapidly removed, postfixed in 4% paraformaldehyde for 1 h, and then cryoprotected overnight (30% sucrose in 0.1 M phosphate buffer, pH 7.4) at room temperature. Serial frozen sections were cut on a cryostat (Reichert-Jung Cryocut 1800) in the coronal plane at a thickness of 24 μm and one every third section was then processed for immunohistochemisty.

The behavioural outcome of the pretreatment with the antagonists, and in particular the latency of the onset of generalised tonic-clonic convulsions from the time of 4-AP injection, was evaluated in parallel experiments, in groups of 15 animals each (75 animals; Table 1).

c-fos mRNA Detection

Six rats were used in this part of the study: three animals were decapitated 1 h following saline injection, and three additional rats were sacrificed 1 h following administration of 4-AP (5 mg/kg);

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: GTS, generalised tonic-clonic seizures; SEM, standard error of the mean.
* $p < 0.05$; ANOVA followed by the *post hoc* Bonferroni test.

the latter group displayed behavioural seizures. Under anaesthesia with diethyl ether, the rats were decapitated, the brains were quickly dissected and samples of the parietal cortex were frozen in liquid nitrogen. Tissue samples were homogenised and the total RNA was extracted by the AGPC method [8]. The reverse transcription (RT) was made from 2 μg RNA as in [60]. One microlitre of the 20 μl 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 [60] and the sequence of c-*fos* primers [4] have been described. Amplification was carried out in 25 cycles after carefully establishing the linearity for both the GAPDH and c-*fos* fragments between 20 and 30 cycles. Identity of the c-*fos* PCR fragment (256 bp) and the GAPDH fragment (377 bp) was confirmed by cloning into 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 normalised to the level of GAPDH mRNA detected from the same amplification reaction.

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$_2$O$_2$ 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), and then in donkey anti-rabbit IgG (1:40; Jackson Immuno-Research, PA). The secondary antibody was detected by the PAP technique (PAP complex diluted to 1:1000). The peroxidase reaction was localised with nickel chloride-containing diaminobenzidine tetrahydrochloride (Sigma), yielding a black reaction product.

Analysis of the Immunohistochemical Data

Quantitative analysis was performed on five sections per animal, selected from every brain on the basis of the same stereotaxic coordinates [43]. Areas of interest (AOIs) for counts of

immunostained neuronal nuclei were selected from the S1Tr region of the neocortex [43], regions CA1, CA2 and CA3 of the Ammon's horn, and from the hilus and granule cell layer of the dentate gyrus [43].

Within each AOI, the immunoreactive cell nuclei were counted using a Nikon Eclipse 600 microscope equipped with a SPOT RT Slider digital camera (1600 × 1200 dpi in 8 bits), using the Image Pro Plus 4 morphometry software (Media Cybernetics, Silver Spring, MD). Following background subtraction, the threshold was determined so that all labelled nuclei could be recognised. The counting was performed blindly of the animal's treatment. The AOIs were determined using the rectangular field of the camera.

In the neocortex, cell counts were done using a 10× objective, and the AOI (an area of 1.2 mm^2) included all neocortical layers (I–VI) from the pia mater to the subcortical white matter (see Fig. 2), so that the layers were not evaluated separately. Cell counts were then normalised to 1 mm^2. In the hippocampus, cell counts were done using a 40× objective, and were again normalised to 1 mm^2. In regions CA1–3, the AOI (an area of 0.05 mm^2) included the stratum pyramidale and a narrow zone of the strata oriens and radiatum. The hilus of the dentate gyrus was outlined according to Amaral [2], 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 labelled cell nuclei were counted in this area. The molecular layer of the dentate fascia contained very few Fos-labelled nuclei and was not evaluated quantitatively.

The data were analysed statistically comparing sets of findings obtained with the same magnification. Differences in the number of Fos-positive cells in the control and the different drug-treated animals were analysed with one-way analysis of variance (ANOVA), followed by the Bonferroni *post hoc* test. A significance criterion of 0.05 was used. The statistical analysis was performed with the SPSS 9.0 software.

RESULTS

Behavioural Analysis

As stated above, groups of animals were destined only to the observation of the behavioural effects of the treatments (Table 1). The i.p. administration of 4-AP caused characteristic behavioural symptoms within 15 min: first, tremor of the vibrissal and masticatory muscles, followed by generalised tremor of the body musculature, detectable as continuous fasciculation of the muscles, and generalised tonic-clonic seizures (GTCS). The symptoms of the GTCS were always sudden and clear-cut, and we could, therefore, easily measure the latency of the GTCS onset (Table 1). Such latency increased significantly in the animals pretreated with ketamine and dextrometorphan, whereas amantadine and MK-801 did not cause any significant change. However, MK-801 pretreatment prevented the development of GTCS in 72.3% of the animals (Table 1). Ketamine exerted a similar, although less pronounced effect: 38.9% of the animals were protected from GTCS. The least effective in this respect was dextrometorphan: only 16.7% of the rats were protected from GTCS (Table 1). Amantadine did not influence instead the seizure symptoms (Table 1). The symptoms preceding GTCS (tremor of the vibrissae, tremor of the masticatory muscles and generalised tremor) were similar in all groups, except for that of the animals pretreated with MK-801. In the latter rats, instead of tremor, the first symptoms were hypotonia of the limbs and unsteady gait, after which generalised tremor also developed. The main behavioural symptoms disappeared after 90–120 min at the latest; the animals then displayed mild tremor or brief myoclonic episodes. By the end of the experiment (3 h), all animals recovered completely. Antagonists given alone did not cause any obvious behavioural change.

The animals used for the immunohistochemistry experiments displayed different seizure symptoms according to the treatment.
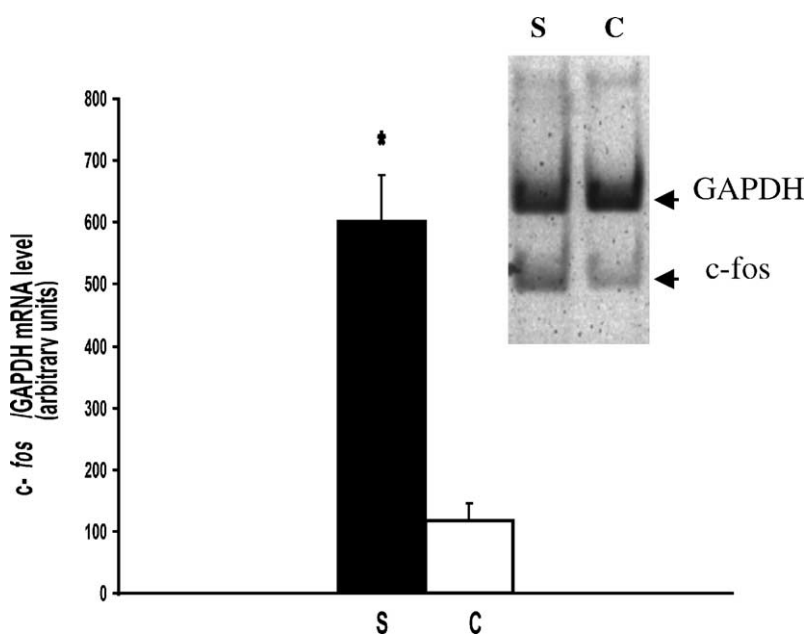

FIG. 1. Level of c-*fos* mRNA in the cerebral neocortex following 4-AP seizures (S, stimulated) and controls (C, control). The columns are the means from three experiments, the vertical bars indicating the standard error of the mean (SEM). The difference is significant (*$p < 0.05$). The inset shows the representative gel of the multiple amplification.

Thus, all of these rats showed generalised tremor, but GTCS developed only in the animals pretreated with amantadine or dextrometorphan. In the ketamine-pretreated group, two animals developed GTCS 60 and 75 min following treatment. None of the MK-801-treated animals displayed GTCS.

Expression of c-fos mRNA

The message of c-*fos* was detectable in the brains of control and 4-AP-treated rats, as was the internal control GAPDH mRNA (Fig. 1). In the control (saline-treated) 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 significantly, from 117 ± 28 arbitrary units (saline-treated) to 602 ± 74 units (4-AP-treated) (Fig. 1).

Immunohistochemistry

Fos-positive cell nuclei were detected in every layer of the neocortex and in the hippocampus 3 h following 4-AP administration. In the neocortex, a large number of Fos-positive cells were distributed in layers II–VI (Fig. 2A). Pretreatment with 40 mg/kg amantadine, 40 mg/kg dextrometorphan, 3 mg/kg ketamine, or 1 mg/kg MK-801 prior to administration of the convulsant agent resulted in a significantly lower number of Fos-immunoreactive nuclei with respect to the non-pretreated animals. Regarding the distribution of labelled cell nuclei, amantadine caused an overall decrease, but strongly stained nuclei were still observed in layers II, III and V (Fig. 2B). Dextrometorphan pretreatment resulted in marked decrease of labelled cells in layers II, III, IV and VI, but a less marked decrease in layer V (Fig. 2C). Ketamine suppressed Fos-like immunostaining in most of the cortical layers, but not in layer IV, which contained numerous Fos-labelled cells (Fig. 2D). MK-801 caused an overall decrease of Fos immunoreactivity, with a staining pattern similar to that observed after dextrometorphan pretreatment (Fig. 2E).

Quantitation revealed that the number of Fos-containing cell nuclei was consistently and significantly lower in animals pretreated with either NMDA antagonist drug than in those treated with 4-AP only (Fig. 4A). It should be reminded, in this respect, that these data were collected across the thickness of the neocortex and not in individual layers (see Fig. 2A–F).
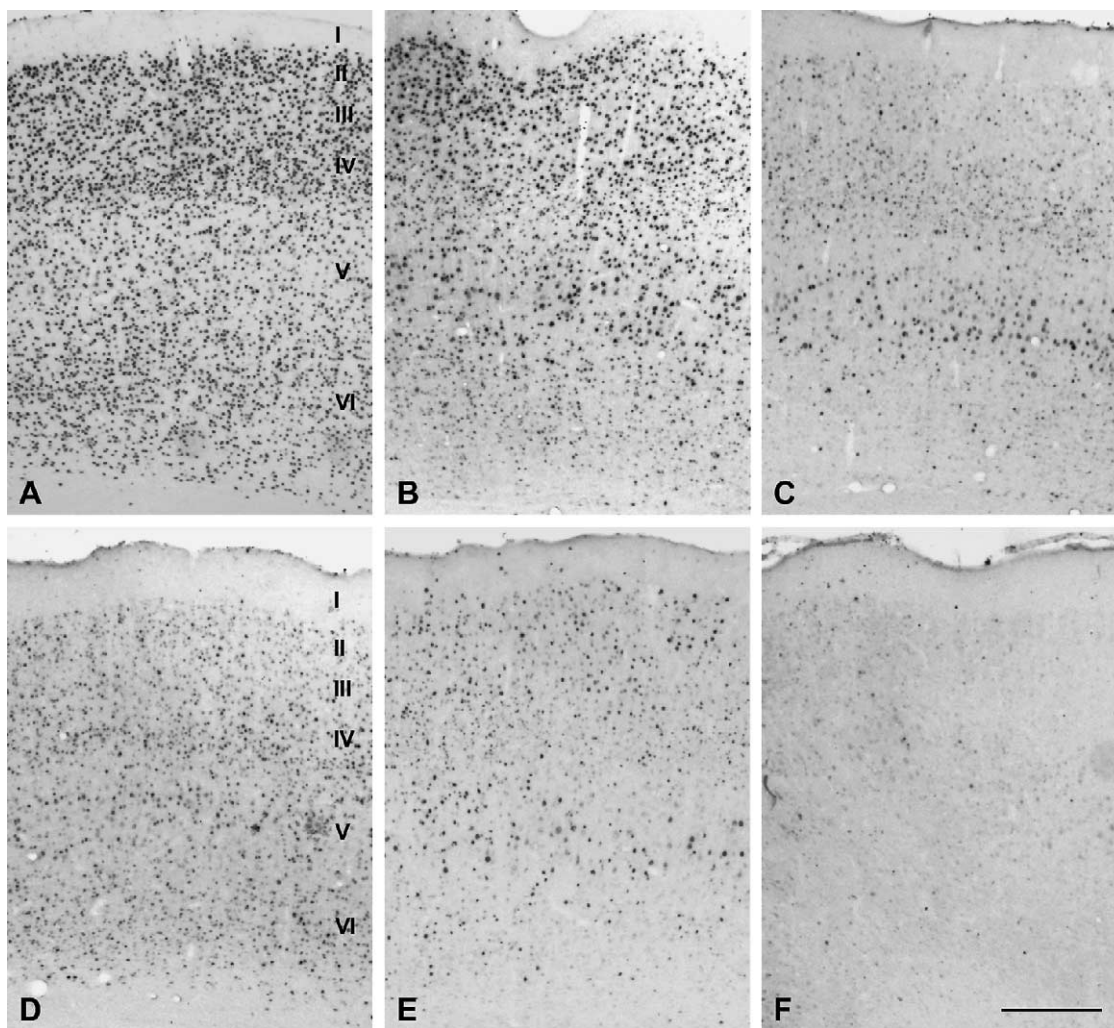

FIG. 2. Low-magnification images of the distribution of Fos-positive cell nuclei in the neocortex 3 h following 4-AP injection. (A) treated only with 4-AP; (B) amantadine pretreatment; (C) dextrometorphan pretreatment; (D) ketamine pretreatment; (E) MK-801 pretreatment; (F) treated only with ketamine (layers are indicated by roman numerals). Bar: 250 μm.

Regions CA1–3 of the hippocampus displayed strong Fos-like staining, mainly in the pyramidal layer. A few scattered nuclei were stained in the strata oriens, radiatum and lacunosum-moleculare. The neurons of the dentate gyrus displayed strong staining, whilst the hilar region contained strongly stained, scattered cell nuclei (Fig. 3A). Pretreatment with amantadine, dextrometorphan, ketamine or MK-801 resulted in a significantly lower number of Fos-labelled neurons in CA1, CA2 and CA3 regions of the Ammon's horn with respect to the animals that had received 4-AP only (Figs. 3B–E and 4C). The less effective among the antagonists was amantadine, which, however, resulted in significant changes in all the above regions, except for CA3 (Fig. 4). Pretreatment with amantadine, MK-801, ketamine or dextrometorphan reduced Fos immunoreactivity in the dentate granule cell layer (Fig. 3B–E), as confirmed by statistical evaluation (Fig. 4B). Moreover, amantadine, ketamine, dextrometorphan and MK-801
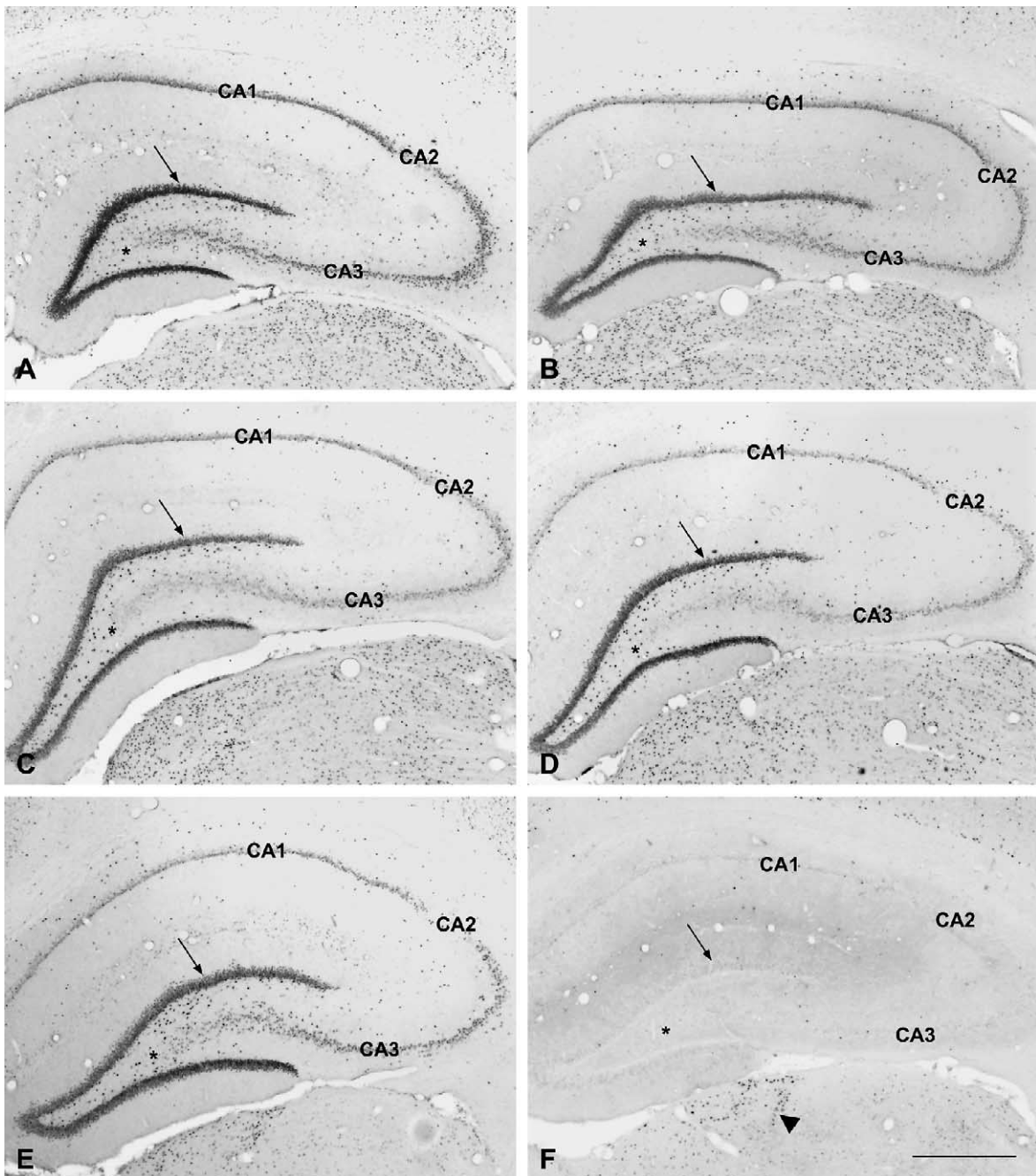

FIG. 3. Low magnification images of the distribution of Fos-positive cell nuclei in the hippocampus. The sectors of Ammon's horn (CA1, CA2 and CA3) are indicated. Arrow points to the granule cell layer of the dentate fascia, while the asterisk shows the hilus of the dentate fascia. (A) treated only with 4-AP; (B) effect of amantadine; (C) effect of dextrometorphan; (D) effect of ketamine; (E) effect of MK-801; (F) treated only with MK-801. The arrowhead in (F) indicates c-*fos* staining in the dorsal thalamus. Bar: 500 μm.
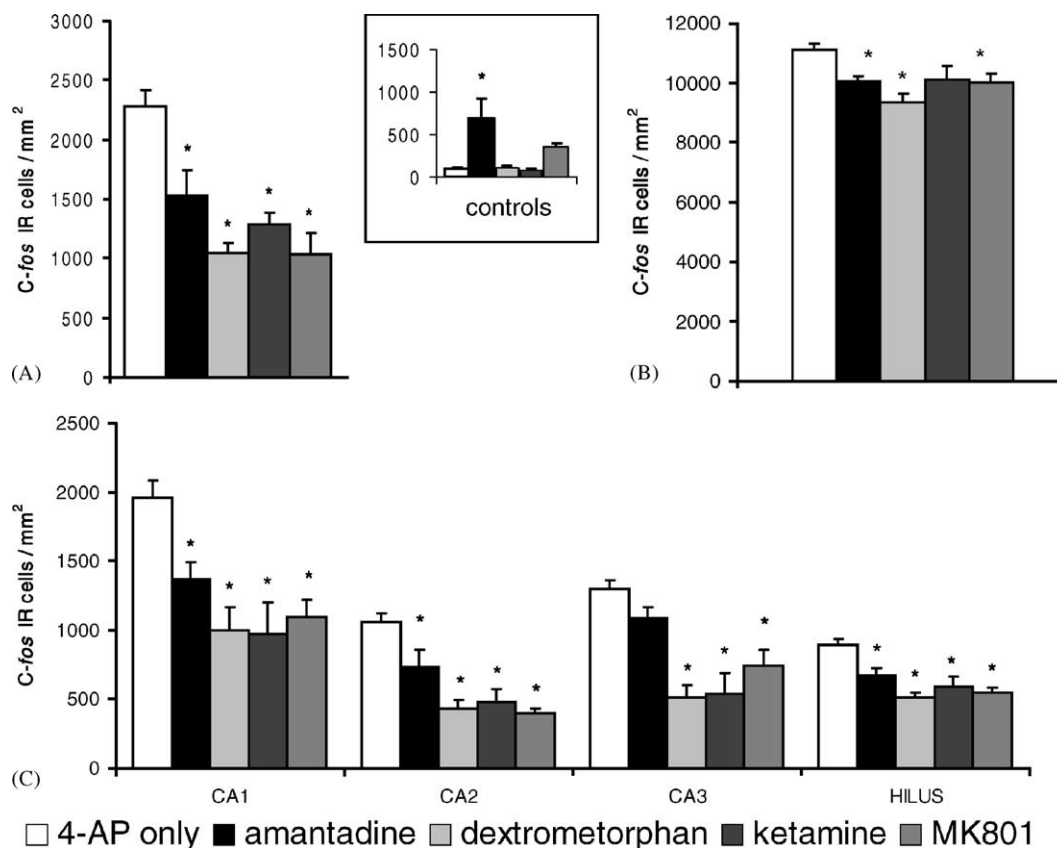
FIG. 4. Quantitative evaluation of Fos-positive cells in the different experimental groups. (A) results of cell counts in the neocortex in amantadine-, dextrometorphan-, ketamine- or MK-801-pretreated animals, compared with rats injected only with 4-AP. The results of cell counts in the neocortex of rats injected only with NMDA antagonists are shown in the "controls" panel (inset). Note that amantadine caused significant c-*fos* expression compared to untreated controls. The first column on the inset diagram displays the number of Fos-stained nuclei following saline injection. Dextrometorphan, ketamine and MK-801 did not cause any significant change. (B) results of cell counts in the granule cell layer of the dentate gyrus in antagonist-pretreated and 4-AP-injected rats compared with animals injected only with 4-AP. (C) results of cell counts in the hippocampus and hilus of the dentate gyrus in antagonist-pretreated and 4-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 in every case; IR, immunoreactive.

resulted in a significant decrease of the number of Fos-containing cell nuclei in the dentate hilus (Fig. 4C).

When given alone, the antagonists caused only minimal cortical Fos induction (Figs. 2F and 3F). In the neocortex, amantadine and MK-801 administration resulted in the relatively highest number of labelled cells, but only amantadine resulted in a significant difference compared to controls injected with saline (Fig. 4A, inset). The number of stained cells induced by MK-801, dextrometorphan and ketamine was consistently very low, without significant differences (Fig. 4A, inset). None of the antagonists did induce a significant increase of Fos expression in the hippocampus (not shown).

DISCUSSION

Previous studies from our laboratory indicated that the 4-AP model is a reliable one for the pharmacological investigation of seizure genesis [34], and that careful counting of the Fos protein-immunostained cell nuclei serves as an indicator of seizure spread in forebrain structures *in vivo* [35,36]. Our previous studies proved that the Fos protein was detectable in the forebrain by Western blotting in 4-AP seizures [37]. In the present experiments, the RT–PCR studies demonstrated the induction of c-*fos* mRNA in the cerebral cortex following 4-AP injection. This is the first such report in the literature.

In a previous investigation [35], strong Fos immunostaining was observed in both the neocortex and the allocortex at 3 h after 4-AP injection, and we therefore chose an interval of 3 h for immunohistochemistry in the present study. On the basis of our evaluation, we conclude that all the NMDA receptor antagonists decreased significantly the seizure-induced expression of c-*fos* in the neocortex and in the allocortex. However, not every antagonist caused symptomatic seizure protection. Our experiments indicated that only ketamine, MK-801 and dextrometorphan attenuated the symptoms of the seizure: ketamine and dextrometorphan increased the latency of the GTCS significantly; MK-801, ketamine and dextrometorphan decreased the incidence of the GTCS. No protective effect was seen in the amantadine-pretreated animals. We, therefore, believe that the immunohistochemical results reflect the antagonistic effect of the drugs on the receptor, and can be correlated only indirectly with the behavioural effects shown by the animals.

Mechanism of Action of Non-Competitive NMDA Antagonists

Ketamine, MK-801, amantadine and dextrometorphan decrease the postsynaptic effects of glutamate mainly by blocking the NMDA receptor channel. The isotope-labelled derivatives of these compounds (except for dextrometorphan) are used in human brain imaging studies to investigate the glutamate receptor function [6].

Ketamine and MK-801 are high-affinity open-channel blockers: they block the ion channel of the receptor at the phencyclidine site and inhibit or decrease the ion fluxes which follow the glutamate binding [7]. MK-801 administration, i.p. and i.c.v., was found to protect against 4-AP seizures in electrophysiological experiments [39]. The present experiments provided further data on the seizure protection, and proved that pretreatments prevent GTCS and reduce c-*fos* induction in the neocortex and hippocampus.

The literature data on the effects of ketamine are not univocal: no effects were seen on the epileptiform activity induced by 4-AP in hippocampal slices [50], but ketamine was found to be effective against picrotoxin seizures *in vitro* [30] and electroconvulsions *in vivo* [52]. Ketamine has been reported to be useful in the therapy of refractory status epilepticus in humans [51]. This drug and its isomers have a proven neuroprotective effect in different conditions such as seizures [9] and ischaemia [46]. In the present experiments, seizure 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 neuron. These effects might be related to the NMDA receptor antagonism of ketamine and its blocking action on Na^+ channels [61].

Amantadine is a low-affinity open-channel antagonist [40]; the family of aminoadamantanes displays antiparkinsonian-like activity as well as neuroprotective action [13]. Memantine (1-amino-3,5-dimethyladamantane) has better therapeutic indices than amantadine and is used in the therapy of epilepsy and other neurological and psychiatric disorders. The present experiments prove that amantadine decreases seizure-induced c-*fos* expression in the neocortex and 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. From these results, we 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, this drug does not provide protection against the behavioural seizure.

The effects of dextrometorphan are more complex. This compound inhibits NMDA-induced convulsions and is, therefore, regarded as a non-competitive antagonist [11,16]. It also diminishes kainic acid seizures and attenuates the consequent hippocampal neuronal damage [27]; it inhibits ischaemia-induced c-*fos* expression and neuronal death [5]. Some of the effects of dextrometorphan are mediated by the NMDA receptors, and some by the voltage-dependent Ca^{2+} and Na^+ channels [55]. The effects of dextrometorphan observed in our experiments are very promising: the decrease in Fos-protein-like immunoreactivity elicited by the seizures in cortical layers indicated that the Ca^{2+} influx was substantially inhibited. Dextrometorphan also increased significantly the latency of the GTCS.

c-fos Expression as Marker of Neuronal Activity

Our findings concerning the appearance of c-*fos* in the convulsing brain are in accord with literature data [14,15,58]. The appearance of synchronised population spikes was found to correlate well with c-*fos* mRNA expression, which also correlated with presynaptic glutamate release [29]. The detection and evaluation of Fos immunoreactivity, therefore, appears suitable for the histological mapping of epileptic neuronal activity [29,35–37]. Literature

data [22,29] lead us to consider 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. Accordingly, decreased seizure-induced c-*fos* expression following the administration of NMDA antagonists should indicate weakening of the postsynaptic effects of glutamate and concomitant influx of Ca^{2+} [20]. However, previous literature data indicated that the NMDA antagonist MK-801 decreased c-*fos* induction significantly, but did not abolish the hippocampal electroencephalographic seizure [29]; this parameter was not examined in the present investigation.

The postsynaptic membranes of neocortical and hippocampal neurons possess not only NMDA, but also AMPA and kainate receptors [31]. These ionotropic receptors respond to extracellular glutamate: AMPA receptors trigger depolarisation and burst initiation, and NMDA receptors become then activated and the opening of the NMDA channel generates large Ca^{2+} influx [25,31]. The blockade of the NMDA channel by non-competitive antagonists obviously inhibits or delays the influx of Ca^{2+}, which in turn should delay or inhibit the long-lasting neuron depolarisation [25]. Since c-*fos* induction is a graded response once it reaches a minimal threshold for expression [29], different c-*fos* expression patterns (resulting in a variation in the number of Fos-positive cells and different staining intensity) are plausible.

It is, however, important to emphasise that GTCS, which represents the main symptom 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 symptom 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 eventually lead to c-*fos* expression [20,29]. Inhibition 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. Another explanation could be based on the role of non-NMDA receptors in the development and maintenance of the symptoms, as supported by literature data [31].

c-fos Expression in the Neocortex and NMDA Antagonists

In vivo studies on the involvement of excitatory amino acid receptors in epileptiform discharges have indicated that NMDA receptors are active in the generation and maintenance of bursting responses in the rat somatosensory cortex [28]. The distribution of ionotropic glutamate receptors is uneven in the neocortex: intracortical synapses of pyramidal neurons are mainly mediated by NMDA receptors [54], whilst the thalamocortical input and the corticothalamocortical neuronal network are rather associated with AMPA receptors [23]. We consider that the significant decrease in the number of Fos-immunopositive cells observed in the present study is a consequence of a reduction in neuronal activity, probably *via* blockade of the NMDA receptor-mediated neurotransmission in the horizontal fibre plexus of the cortex and in other corticocortical synapses [19]. On the other hand, the AMPA-mediated [23] thalamocortical pathway remains active. This activity was most apparent in the cases pretreated with ketamine and MK-801, in which layer IV exhibited relatively high Fos immunoreactivity, indicating the importance of non-NMDA-mediated stimulatory effects of thalamocortical axons. The results obtained with amantadine indicated that this drug was probably loosely bound in the NMDA channel,

and it was removed by the intense cation fluxes during neuron hyperactivity [40]. The persistence of the motor seizure symptoms following amantadine pretreatment supports this assumption.

c-fos Expression in the Hippocampus and NMDA Antagonists

Literature data lead us to assume that the limbic seizures produced by 4-AP are related to the enhancement of glutamatergic transmission [35,44]. Antagonists of the NMDA receptor inhibit seizure-induced c-fos mRNA expression in the dentate granule cells [29], suggesting that dentate neuronal hyperactivity and thereby c-fos induction are mediated by NMDA receptor activation. Furthermore, competitive and non-competitive NMDA receptor antagonists prevented 4-AP seizure-induced neurodegeneration in the CA1 and CA3 regions with an NMDA-mediated excitotoxic mechanism [44]. However, both NMDA and non-NMDA receptors play a role in the epileptiform discharges mediated by the perforant path in the dentate gyrus [26]. Immunohistochemical studies have confirmed the presence of AMPA receptor subunits in the molecular layer of the dentate gyrus [38]. 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 protein-like staining in the Ammon's horn after pretreatment with NMDA antagonists indicated the importance of NMDA-mediated glutamate action in the maintenance of the seizure, the remaining Fos immunoreactivity highlighted the importance of non-NMDA glutamate receptors, and probably other transmitter systems, in epileptogenesis.

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