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COMPETITIVE AND NON-COMPETITIVE NMDA RECEPTOR ANTAGONISTS INDUCE c-Fos EXPRESSION IN THE RAT ANTERIOR, CINGULATE CORTEX

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In the present study we tried to find out whether the competitive NMDA receptor antagonist CGP 40116 was capable of inducing c-Fos expression in the rat cingulate cortex in a manner similar to that described previously for the non-competitive NMDA receptor antagonist MK-801. Induction of fast early genes by MK-801, especially in the rat cortex, has recently been linked with the neurotoxic effects of non-competitive NMDA receptor antagonists on cortical neurones, hence it was of interest to extend those studies to another class of NMDA receptors antagonists i. e. competitive one. It was found that CGP 40116 (2.5 and 5 mg/kg) induced c-Fos expression in the rat anterior cingulate cortex. That effect was dose-dependent and was shown as an increase in the number of cells expressing the c-Fos immunoreactivity. A qualitatively similar, but quantitatively stronger, effect was observed after administration of MK-801 (0.2 and 0.4 mg/kg), which also caused a dose-dependent increase in the number of c-Fos positive neurones. The described dose-dependent effects of CGP 40116 and MK-801 are shown as an increase in the number of c-Fos-positive nwurones, but not as an increase in the optical density of c-Fos immunostaining in c-Fos positive neurones. In control, vehicle-injected rats, the constitutive c-Fos immunoreactivity was not found in the rat anterior cingulate cortex. The obtained data indicate that both competitive and non-competitive NMDA receptor antagonists may induce similar effects on the c-Fos immunoreactivity in the rat anterior cingulate cortex, and that their administration may lead to similar functional consequences resulting form activation of fast, early genes.

Key words: *c-Fos-immunoreactivity, cingulate cortex, NMDA antagonist, MK-801, CGP 40116, rats*

INTRODUCTION

In recent years several published reports have suggested that drugs operating via NMDA receptors in experimental animals may evoke effects indicative of their therapeutic potentials (1—3). On the other hand, there also exists evidence that non-competitive NMDA receptor antagonists, such as

MK-801 or phencyclidine, evoke neurotoxic and psychotomimetic effects (3—5). The lack of consistent data showing whether competitive NMDA antagonists possess or are devoid of similar side-effects prompted several studies to compare the effects of both types of NMDA receptor antagonists in experimental models used to illustrate their psychotomimetic and neurotoxic properties (3). Therefore we think that a quantitative comparison between the effect of MK-801 (non-competitive) and CGP 40116 (competitive NMDA receptor antagonists) on the induction of c-Fos protein in the rat anterior cingulate cortex is in line with the above-mentioned experimental trend. Activation of immediate early gene c-fos and production of c-Fos protein in response to a variety of physiological and pharmacological manipulations has been widely used as a marker of neuronal activation (6). The available data indicate that MK-801 evokes a massive induction of c-Fos proteins, mainly in the rat posterior cingulate/retrosplemnial cortex (7—10) an effect which is postulated to be associated with not only neurotoxic, but also psychotomimetic (4, 5, 11—13) properties of non-competitive NMDA antagonists operating *via* the phencyclidine binding site of the NMDA receptor ion channel complex (14). Taking into account the fact that the anterior cingulate cortex was also a target for MK-801 (5, 12, 13), we thought that our experiment on the c-Fos expression in that region of the cortex would help not only to compare the activity of competitive and non-competitive NMDA receptor antagonists in another brain region, but also to show their potential psychotomimetic properties as a consequence of changes in the neuronal activity, measured by that procedure. In the present study we chose CGP 40116 as a competitive NMDA receptor antagonist, since it is a highly selective NMDA receptor antagonist (plus isomer of CGP 37849), active centrally after its peripheral administration (14). MK-801 was used as a representative of non-competitive NMDA antagonists and as a reference drug with a potent impact on c-Fos immunoreactivity in the rat posterior/retrosplemnial cortex (7—10).

MATERIALS AND METHODS

All the experiments were carried out on Wistar male rats weighing 200—250 g. The rats were housed in groups (6 animals per cage) on an artificial light/dark cycle (12/12 hours, light on at 07:00), with free access to standard laboratory food (LSM, Bacutil) and tap water, and were extensively handled for one week prior to further studies. Two hours after administration of different doses of MK-801 or CGP 40116 (0.2, 0.4 and 2.5, 5 mg/kg i.p., respectively), the rats (6 animals per dose of a given drug) were deeply anaesthetized with sodium pentobarbital (100 mg/kg) and were transcardially perfused with saline followed by a 4% paraformaldehyde in a 0.1 M phosphate buffer saline (PBS). Following a 24-hour postifixation period, 40 μm thick sections were cut at the level of the anterior cingulate cortex using a vibratome (Technical Products International); sections were selected according to a stereotactic atlas of Paxinos and Watson (15), (planes 3.7—2.0 mm from bregma). Free-floating sections of the rat brain were washed tree times with a 0.1 M phosphate buffer (PBS, pH = 7.4) before further incubation for 10 min in PBS

containing a 0.3% hydrogen peroxide in order to eliminate the activity of endogenous peroxidase. The sections were then washed with PBS (three times, for 10 min) and incubated for 60 min with a 5% normal rabbit serum (Vector Lab) in PBS containing 0.1% Triton X-100. Then the sections were incubated with primary c-Fos antisera (dilution 1 : 2000, containing a 1% Triton X-100, and a 0.03% sodium azide) for 48 hours at a temperature of 4°C. A polyclonal sheep antibody (Cambridge Research Biochemical, CRB OA-11-823), directed against residues 2—16 of the n-terminal region of c-Fos proteins, was used in the present experiment. After incubation with primary antiserum, the sections were washed tree times with PBS and incubated with a biotynilated, secondary rabbit-antisheep antibody (Vector Laboratories, dilution 1 : 500; in PBS containing a 0.1% Triton X-100; the time of incubation = 60 min). The final incubation of the sections for 60 min with a solution of the avidin-biotinilated horseradish peroxidase complex (0.5%; Vector Lab; diluted in PBS containing a 1% Triton X-100), was preceded by washing with PBS (three times, for 10 min each). The reaction was visualized by a glucose oxidase-diamino-benzidine nickel method. In order to minimise variations in the staining, in each experiment the brain sections obtained from vehicle-, MK-801 — and CGP 40116-treated rats were stained for the c-Fos immunoreactivity in parallel. c-Fos-positive nuclei were counted with a NIKON OPTIPHOT microscope, equipped with a computerised image analysis system (Java, Jandel) and a microphotographic system (NIKON UFX-DX). The number of c-Fos positive neurones was assessed in the region of anterior cingulate cortex using a standard 370/420 μm frame. Additionally, the optical density of each individual c-Fos-positive neurone was measured using the above image analyser. A Kodak grey scale was used as an external standard to normalise the background staining in different sections and experiments.

RESULTS

It was found that the competitive NMDA receptor antagonist CGP 40116, given in doses of 2.5 and 5 mg/kg, induced a dose-dependent increase in the number of neurones expressing c-Fos immunoreactivity in the rat anterior cingulate cortex (*Fig. 1*). A similar effect was also observed after administration of MK-801, a non-competitive NMDA receptor antagonist, which — when given in doses of 0.2 and 0.4 mg/kg — evoked the appearance of c-Fos-positive neurones, in proportion to the applied dose (*Fig. 2*). In control, vehicle-treated animals, no c-Fos-positive neurones were found in that brain region. Interestingly, the optical density of immunostatinig of c-Fos-positive neurones was independent of the applied doses of either drug (*Fig. 3*). The observed profile of action on the optical density does not depend on the total saturation of the staining reaction, since c-Fos-positive neurones showed a wide range of optical density (*Fig. 3*). Thus the apparent lack of effect on the density of immunostaining and the concomitant dose-dependent increase in the number of c-Fos-positive cells may indicate that the effectiveness of competitive and non-competitive antagonists of NMDA receptors on c-Fos expression in the rat anterior cingulate cortex is associated with recruitment of a greater amount of c-Fos-positive neurones, and not with the amount of c-Fos proteins in individual neurones. After both those drugs, c-Fos proteins were found in all layers of the rat anterior cingulate cortex, the higher density being observed in its medial part.

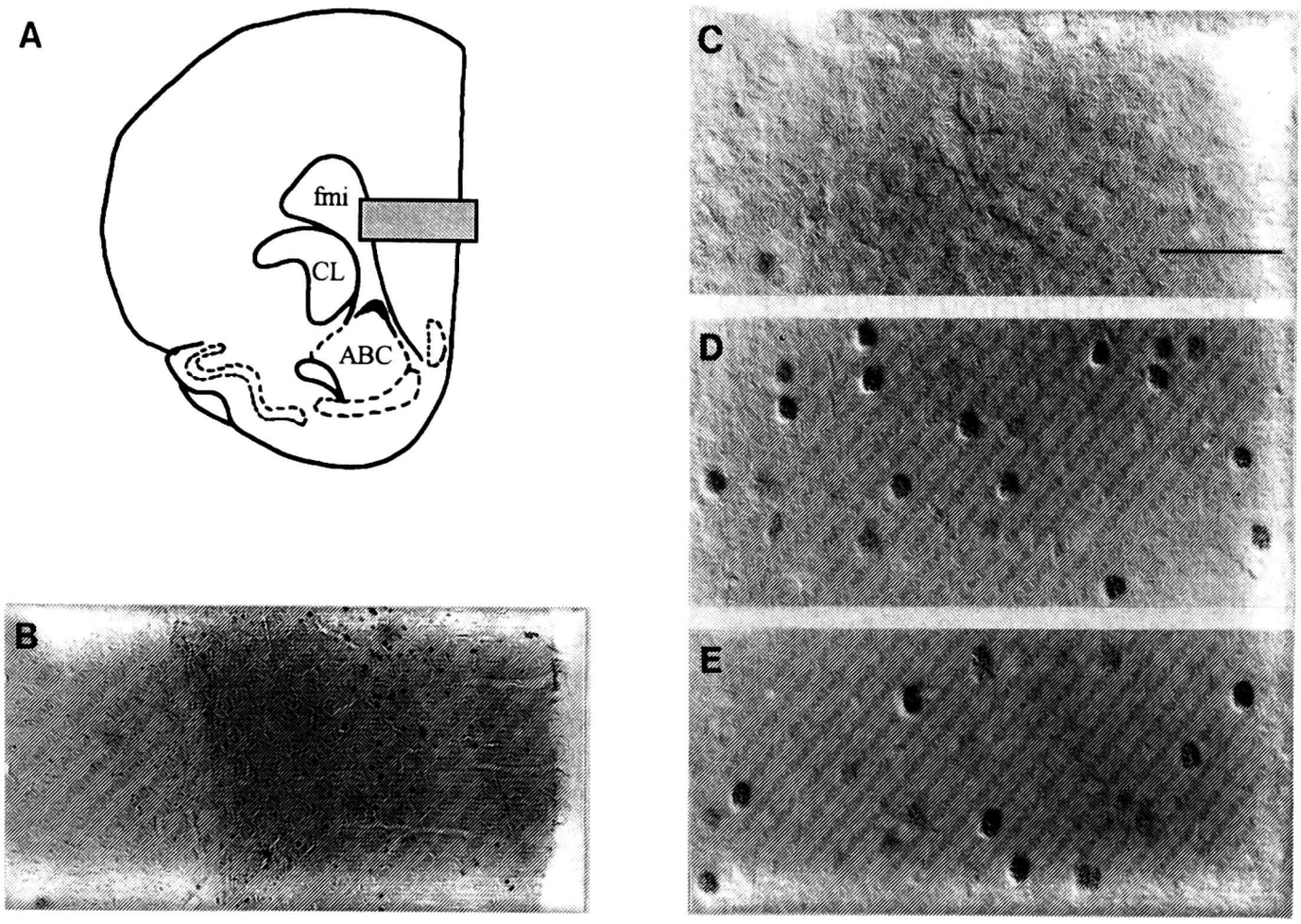

Fig. 1. Representative distribution of c-Fos positive nuclei, labelled immunocytochemically in the rat anterior cingulate cortex. A — schematic representation of area selected for quantification of MK-801 — and CGP 40116-induced expression of c-Fos. B — distribution of c-Fos positive nuclei in the anterior cingulate cortex after administration of MK-801 (0.4 mg/kg). C, D, E — high power photomicrographs (20 X objective, Nomarski optic) illustrating respectively: (C) effects of vehicle, (D) MK-801 (0.4 mg/kg), and (E) CGP 40116 (5 mg/kg) on expression of c-Fos in anterior cingulate cortex. **Abrevations:** ABC — nucleus accumbens septi; CL — claustrum, fmi — forceps minor corpus callosum.

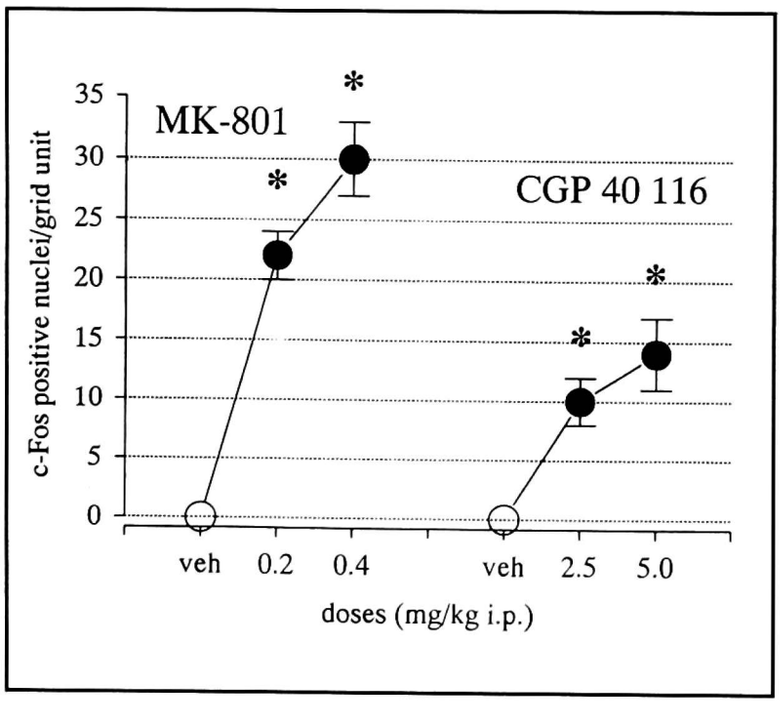

Fig. 2. The number of c-Fos-positive nuclei within a 370×420 μm: area in the anterior cingulate cortex in control (open symbols) and MK-801 — or CGP 40116-treated rats (filled symbols). MK-801 (0.2 and 0.4 mg/kg i. p.) and CGP 40116 (2.5 and 5.0 mg/kg i. p.) were given 2 hours prior to the perfusion. The values shown represent group mean $\pm$ S.E.M. Asterisks denote statistical differences between the number of objects in control (vehicle injected) and drug-treated rats; Dunnett's test after one way ANOVA, $p < 0.05$.

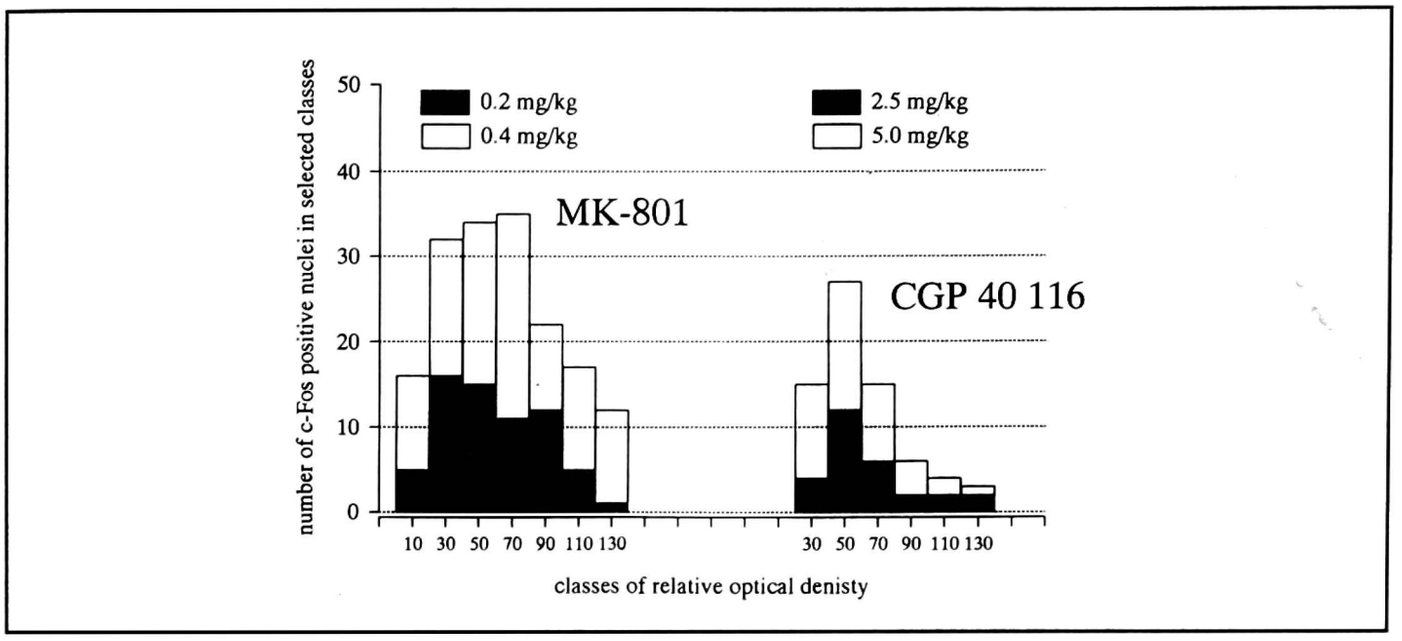

Fig. 3. The distribution of relative optical density of c-Fos-positive nuclei in MK-801 — and CGP 40116-treated rats, measured in the anterior cingulate cortex. Each bar represents the number of c-Fos-positive nuclei in the arbitrary selected classes of optical density, ranging from 256 (white) to 0 (black) minus the density of the background. The background was measured within the close proximity of every single nucleus and was subtracted from the density of given nucleus. Open and filled bars illustrate the results obtained after lower (0.2 or 2.5 mg/kg) and higher (0.4 and 5.0 mg/kg) doses of MK-801 and CGP 40116 (left and right panels respectively). Multiple CHI-square test.

DISCUSSION

Our present study indicates that CGP 40116 and MK-801 induce c-Fos expression not only in the posterior cingulate-retrosplemnial cortex (7, 8, 10) and thalamus (6), (as was shown by others authors), but also in another part of cortex, i.e. the anterior cingulate cortex. The observed similarities between competitive and non-competitive NMDA receptor antagonists at the level of c-Fos expression may indicate that a functional blockade of the information flow *via* different entities of the NMDA receptor, such as a phencyclidine binding site and a receptor recognition site (3), may lead to similar functional consequences resulting from activation of fast early genes. Importantly, our study demonstrates the effects of both these antagonists at the doses used in experimental models to display their therapeutic potentials while the above-cited authors reported the effects of MK-801 on c-Fos expression at doses even as high as 5 mg/kg, which induce an anaesthetic effect in rats (7—10). Thus our results suggest that activation of cortical neurones, seen as appearance of c-Fos proteins, may be obtained in the anterior cingulate cortex after administration of behaviourally relevant doses. The appearance of c-Fos expression in the rat posterior cingulate/retrosplemnial cortex after MK-801 has been linked with activation of cortical pyramidal neurones, which at the

proximate state of activation undergo reversible injury (1, 2, 10, 16). The latter hypothesis is supported by the observation that MK-801, as well as phencyclidine and other non-competitive NMDA receptor antagonists induce the appearance of heat shock proteins (HSP70) — a cellular markers of non-lethal neuronal stress (16) and parallely these substances cause vacuolisation of neurones in the cingulate cortex (1, 2). With the respect to c-Fos expression and its possible link with the final neurotoxic effect, a similar impact of CGP 40116 and MK-801 on the anterior cingulate cortex may support some earlier suggestions that these two classes of NMDA receptor antagonists may produce similar side-effects (3). Additionally, our data seem to suggest that the occurrence of the above effects after administration of CGP 40116 and MK-801 may take place even when these drugs are given in behaviourally relevant doses.

Apparent activation of cortical neurones, seen as c-Fos expression, may be explained by the mechanisms proposed by Onley et al. (1, 2) (*Fig. 4*) in order to interpret the neurotoxic effects of competitive and non-competitive NMDA antagonists. It is proposed that cortical pyramidal neurones are under inhibitory influence of cortical GABA-ergic interneurones which have NMDA receptors. The release of excitatory amino acids from axons collateral of pyramidal neurones, subsequent to the activation of pyramidal cells, will facilitate the inhibitory impact of GABA neurones on cortical pyramidal output cells. Conversely, NMDA receptor antagonists will abolish the inhibitory effects of GABA interneurones by the blockade of NMDA receptors located on GABA-ergic interneurones which, in consequence, will lead to activation of pyramidal cells — seen, for example as the appearance of c-Fos proteins and — in the proximate state of activation — to a reversible injury of neurones seen as the appearance of vacuolisation and HSP 70 (16). Indeed, some available data demonstrate that the appearance of c-Fos after administration of MK-801 might be associated with neuronal degeneration as was shown in experiments using the mRNA encoding synthesis of c-Fos proteins together with BDNF, a marker of neuronal injury (10). However the above concepts, should be put forward carefully, since in the present study we report appearance of c-Fos proteins in the anterior cingulate cortex, whereas the above-cited hypothesis explains the effects observed in the posterior cingulate/retrosplemnial cortex. Further studies, defining type of cortical cells expressing c-Fos and experiments investigating whether mechanisms operating in the cingulate/retrosplemnial cortex are also operative in the cortical region selected for the present study, will be necessary to verify this hypothesis.

Interestingly enough, the permanent hyperactivity of cortical pyramidal neurones has recently been associated with the pathophysiology of schizophrenia since some findings indicate neurodegenerations of cortical GABA-ergic neurones in the brains of schizophrenics (11). Thus, in functional

terms the effects seen after administration of NMDA antagonists which turn off the inhibitory effects of GABA interneurones on pyramidal neurones, may lead to similar psychotomimetic and pathophysiological consequences which are found in the course of schizophrenia (11). The latter speculation, is in line with the findings that competitive and non-competitive NMDA antagonists have psychotomimetic properties both in humans and experimental animals, which is the main limiting factor of their clinical application. Secondly, it may suggest that, the effects of NMDA antagonists at least in some subregions of the cingulate cortex may be an interesting model of psychosis.

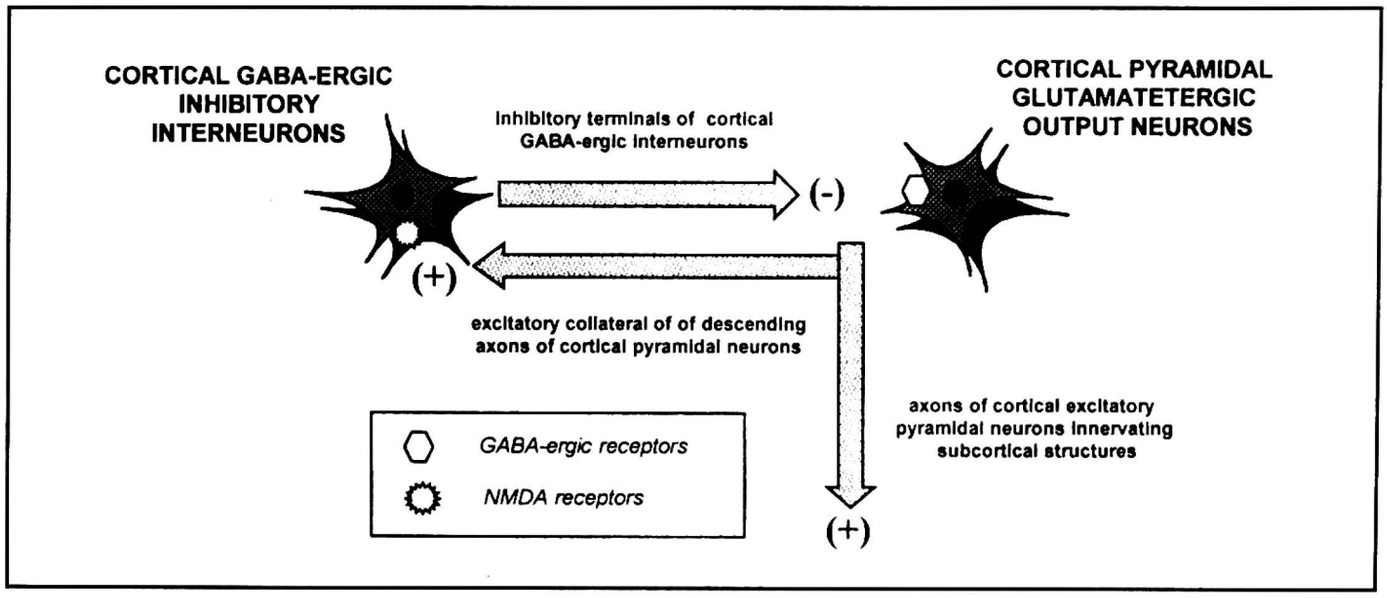

Fig. 4. Model of cortical pyramidal stimulation after administration of MK-801 and CGP 40116. Pyramidal neurones have GABA-ergic receptors. An axon collaterals activate the NMDA receptors on cortical GABA-ergic inhibitory interneurones. Cortical pyramidal glutamatergic neurones have GABA-ergic receptors. MK-801/CGP 40116 blocks NMDA receptors on GABA-ergic interneurones, what attenuates the process of tonic inhibition of pyramidal neurones.

The functional consequences of the observed c-Fos expression in the rat anterior cingulate cortex are only speculative and they cannot be linked exclusively with the pathology of neurones in the cingulate cortex, but also have to be referred to other brain regions, innervated by afferents of the cingulate cortex (17—19). For example, it is known that glutamineric afferents of the cingulate cortex (18, 19) are involved in regulation of the level of dopamine receptor stimulation in subcortical dopaminergic brain structures (11, 20—23). Moreover, these neuronal circuits are likely to be involved in the pathophysiology of psychotic states (11) and in the mechanism of sensitisation (12, 22). Finally, they are supposed to regulate the set point of responsiveness of experimental animals to dopamine-mediated physiological and pharmacological stimuli in subcortical dopaminergic structures (21—23). Indeed, several reports have indicated that both competitive and non-competitive NMDA receptor antagonists enhance the responsiveness of

rats and mice to dopaminergic agonists (24, 25) and evoke alterations in the density of dopaminergic receptors (13). Further studies will be required to link more directly the alterations in the activity of cortical neurones, regarded as an expression of c-Fos, with the already available behavioural and biochemical data. However, it is obvious that any future theory attempting to explain the interaction between NMDA antagonists and the dopaminergic neurotransmission should deal with alterations in the cingulate cortex neurones, evoked by both types of NMDA receptor antagonists which, in turn, regulate the functions of subcortical dopaminergic systems.

CONCLUSIONS

Summing up, our data provide further evidence that, apart from non-competitive NMDA antagonists, also competitive ones, such as CGP 40116, induce expression of c-Fos immunoreactivity in the rat anterior cingulate cortex. It is speculated that the latter effect may be connected with psychotomimetic properties of competitive NMDA antagonists, as was found previously for competitive and non-competitive NMDA receptor antagonists in differenet regions of the cortex, and may be associated with the pathophysiology of cortico-subcortical glutamatergic pathways.

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