Excitatory Actions of NMDA Receptor Antagonists in Rat Entorhinal Cortex and Cultured Entorhinal Cortical Neurons

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We have characterized excitatory effects of non-competitive NMDA receptor antagonists MK-801, PCP, and ketamine in the rat entorhinal cortex and in cultured primary entorhinal cortical neurons using expression of immediate early gene c-fos as an indicator. NMDA receptor antagonists produced a strong and dose-dependent increase in c-fos mRNA and protein expression confined to neurons in the layer III of the caudal entorhinal cortex. Induction of c-fos mRNA is delayed and it is inhibited by antipsychotic drugs. Cultured entorhinal neurons are killed by high doses of MK-801 and PCP but c-fos expression is not induced in these neurons indicating that this in vitro model does not fully replicate the in vivo effects of PCP-like drugs in the entorhinal cortex. Excitatory effects of the NMDA receptor antagonists may be connected with the psychotropic side effects of these drugs and might become a useful model system to investigate neurobiology of psychosis.
[Neuropsychopharmacology 21:137–146, 1999]
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NMDA receptor antagonists, such as phencyclidine, ketamine, and MK-801 (dizocilpine) produce in humans psychotogenic side effects which clinically closely resemble schizophrenia and are characterized by distorted body image, hallucinations, vivid dreams, and delirium (Johnson and Jones 1990; Javitt and Zukin 1991). Ketamine also frequently induces psychotic "emer-

gence reactions" in adults (Krystal et al. 1994). Because of these parallels between the psychotomimetic state produced by the NMDA receptor antagonists and schizophrenia, PCP-induced psychosis has widely been used as an experimental model of schizophrenia (Javitt and Zukin 1991).

In a series of pioneering studies, Olney and coworkers demonstrated that NMDA receptor antagonists produce reversible neurotoxic effects in neurons in the posterior cingulate and retrosplenial cortices (RSC) of rat brain (Olney et al. 1989, 1991; Olney and Farber 1995a, 1995b). These effects are characterized by reversible intracellular vacuolization (Olney et al. 1989) and induction of mRNAs for c-fos (Dragunow and Faull 1990; Hughes et al. 1993; Näkki et al. 1996), heat shock protein 70 (HSP70) (Sharp et al. 1991, 1994; Näkki et al. 1995), and brain-derived neurotrophic factor (BDNF) (Castrén et al. 1993; Hughes et al. 1993). Induction of c-fos and BDNF is typically observed in activated neurons and we therefore tentatively call these effects "ex-

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Received September 21, 1998; revised December 14, 1998; accepted January 20, 1999.

NEUROPSYCHOPHARMACOLOGY 1999–VOL. 21, NO. 1
© 1999 American College of Neuropsychopharmacology
Published by Elsevier Science Inc.
655 Avenue of the Americas, New York, NY 10010

0893-133X/99/$–see front matter
PII S0893-133X(99)00006-8

citatory" to distinguish them from the prevention of excitation, which is the typical effect of NMDA receptor antagonists in other brain areas. These excitatory actions may be connected with the psychotomimetic side effects produced by PCP-like drugs (Olney and Farber 1995a, 1995b). We have previously observed that MK-801 increases mRNA for BDNF not only in the retrosplenial cortex but also in the entorhinal cortex (EC) (Castrén et al. 1993). EC is the input and output station of the information going to and coming from the hippocampus (Jones 1993; Amaral and Witter 1995). It is thought that the EC-hippocampus loop is very important in the processing of sensory information and defects in its normal function might produce cognitive disturbances (Jones 1993). Therefore, neurons in the entorhinal cortex are of particular interest in the studies directed at the pathophysiology of schizophrenia.

Morphological and imaging studies have also implicated disturbances in the entorhinal cortex in the pathophysiology of schizophrenia. Analysis of post-mortem material have suggested that brains of schizophrenic patients may display cytoarchitectonic disturbances in the entorhinal cortex (Falkai et al. 1988; Arnold et al. 1991; Bogerts 1993; Jakob and Beckmann 1994). CT scans of brains of schizophrenic patients have consistently demonstrated loss of gray matter in parahippocampal gyrus (Roberts 1990). Furthermore, a recent study where schizophrenic patients were scanned in PET scan while they experienced hallucinations found increased neuronal activity in the entorhinal cortex associated with hallucinations (Silbersweig et al. 1995). Unfortunately, the spatial resolution of imaging studies is not high enough to localize the neuronal population that is the source of aberrant activity and it is very difficult to identify the normal identity of the disturbed neurons in post-mortem morphological studies. It would therefore be very important to find methods that could recognize neuronal populations that are disturbed in psychotic symptoms.

NMDA receptor antagonists may mimic a pathological process which takes place in all or a subpopulation of the PCP-sensitive neurons during a schizophrenic psychosis. Therefore, treatment of experimental animals with NMDA antagonist may help to identify neurons which mediate psychotic behavior and allow their cellular and molecular properties to be investigated. In this report we have investigated and characterized the excitatory actions of NMDA-receptor antagonists in the rat EC using c-fos expression as a reporter system. c-fos is an immediate early gene that is rapidly and transiently induced by numerous stimuli that activate neurons, such as depolarization and seizure activity and induction of c-fos expression has widely been used as a marker of cellular activation in the nervous system (Morgan and Curran 1991). We have also investigated whether cultured neurons dissociated from embryonic

rat entorhinal cortex display similar properties as EC neurons in vivo and whether these cells could be used in antipsychotic drug development and as a possible cellular model in schizophrenia research.

MATERIALS AND METHODS

Male Wistar rats (178–211 g) were injected intraperitoneally (IP) with MK-801 (Research Biochemicals Inc., Natick, MA) in doses of 0.1, 0.5, 1.0, 2.0, and 5.0 mg/kg and allowed to survive for 4 hours ($n = 22$, two rats in 0.1-mg group, four in other treatment groups). Additional male animals received an ip injection of MK-801 (5 mg/kg) and were sacrificed 1/2, 1, 2, 4, 8, 12, 24, and 48 hours after the injection ($n = 4$ for each group). Four control animals received an equal volume of 0.9% saline and were sacrificed 4 hours after the injection. In addition, rats (181–202 g) were injected with ketamine (10 mg/kg, 25 mg/kg, and 50 mg/kg, ip, Parke-Davis) or PCP (15 mg/kg, Sigma, St. Louis, MO) and allowed to survive for 4 hours (three rats in each group). To investigate the possible inhibitory effects of antipsychotic drugs, clozapine (Sandoz AG, Nürnberg, FRG, 25 mg/kg), haloperidol (Sigma, 2 mg/kg) and desipramine (Sigma, 15 mg/kg) were injected into four rats each 60 minutes before MK-801 injection and rats were sacrificed 4 hours afterwards. For apoptosis studies rats (at least two rats in each group) were injected by various doses of MK-801 up to 10 mg/kg and rats were sacrificed after 24 hours. Animals were anesthetized with carbon dioxide, decapitated, and the brains were removed and frozen on isopentane and dry ice. Rats used for immunohistochemistry were deeply anesthetized with an overdose of pentobarbital and transcardially perfused with buffered 4% paraformaldehyde. Dissected brains were postfixed overnight in the fixative and overnight in 20% buffered sucrose, frozen and sectioned to 12-μm thick horizontal sections in a Jung CM 3000 cryostat.

In situ hybridization was performed as previously described (Lindholm et al. 1993). An anti-sense cRNA probe against rat c-fos was labeled with ^{35}S-UTP (NEN) using SP6 RNA polymerase and purified over Nick Columns (Pharmacia Biotech). Sections were fixed in 4% paraformaldehyde/PBS at 4°C, washed and 150 μl of the hybridization solution (containing 1.0×10^6 cpm/ml of the probe, 120 mM dithiotreitol, 500 μg/ml tRNA, 50% formamide, 10% Dextran SO_4, 0.3 M NaCl, 1X Denhardt's reagent, 2 mM Tris, 1 mM EDTA) was placed between two slides and the slides were sandwiched together. After 12 hours at 60°C, sections were washed 4×10 minutes in $1 \times$ SSC and treated with RNaseA (20 μg/ml of RNase A, Boehringer), at 37°C for 30 minutes. Sections were then washed in increasing stringency up to $0.1 \times$ SSC at 60°C, dehydrated in ethanol, air dried

and exposed to Kodak X-Omat film for 6 days or to autoradiography emulsion (Kodak NTB2) for 4 weeks. The optical density in scanned x-ray films was measured by using NIH Image (version 1.52) software.

For immunohistochemistry, sections were permeabilized in Triton X-100, incubated overnight at +4°C with an anti-c-fos antibody (Santa Cruz Biochemicals), washed and incubated for 2 hours with an anti-rabbit secondary antibody. The staining was visualized using the peroxidase-antiperoxidase reaction (ABC kit, Vector laboratories). Omission of the first antibody produced no visible staining reaction (data not shown).

Cortical neurons from area adjacent to hippocampus which includes EC, were dissected from E17 rat embryos and prepared for culture as described (Lindholm et al. 1993). MK-801, PCP, pentobarbital (Sigma) and BDNF (50 ng/ml, Alomone laboratories, Israel) haloperidol (1 μM) and chlorpromazine (Sigma, 1 μM) were added on the fifth day in culture at indicated doses and live neurons (which exclude trypan blue) were counted 2 days after exposure under phase-contrast microscopy. Induction of c-fos expression was determined by immunohistochemistry in neurons fixed in 4% buffered paraformaldehyde at different times after the addition of MK-801 or PCP using a polyclonal anti-c-fos antibody. Results are expressed in mean ± SEM. ANOVA and Student's *t*-test were used for statistical analysis and a *p*-value smaller that .05 was considered statistically significant.

RESULTS

The effects of MK-801, PCP, and ketamine in rat entorhinal cortex   MK-801 induced a dramatic increase in c-fos mRNA in the EC at 4 hours after the administration (Figure 1). PCP at the dose of 15 mg/kg also induced c-fos mRNA expression in the same anatomical area (Figure 1). Ketamine at the dose of 50 mg/kg produced an increase in c-fos mRNA which was indistinguishable from the effect of MK-801 (Figure 1), but lower doses of ketamine (10 and 25 mg/kg) did not influence c-fos mRNA in the EC (not shown). c-fos protein was strongly induced in the same anatomical area at 6 hours following MK-801 injection (Figure 2).

Horizontal sections through the EC revealed a striking compartmentalization of the increase in c-fos mRNA and protein. The increase was by far most pronounced in the medial EC (mEC) (Figures 1 and 2) but a smaller number of strongly c-fos immunoreactive neurons could be seen also in the lateral EC (Figure 2A). The effect was also essentially restricted to layer III of the mEC. In this layer, the great majority of cells displayed strong increase in c-fos mRNA and protein levels (Figures 1 and 2). There was a more restricted increase in some neurons in layer VI but no c-fos mRNA induction

was detectable in other layers of the mEC. In saline-treated rats, light c-fos immunoreactivity could be observed only in few scattered neurons (Figure 2B and 2E).

Analysis of c-fos mRNA expression after MK-801 injection in serial horizontal sections cut through the EC revealed a strong band of c-fos mRNA positive cells dorsally at the level where the EC begins (Figure 3) [see
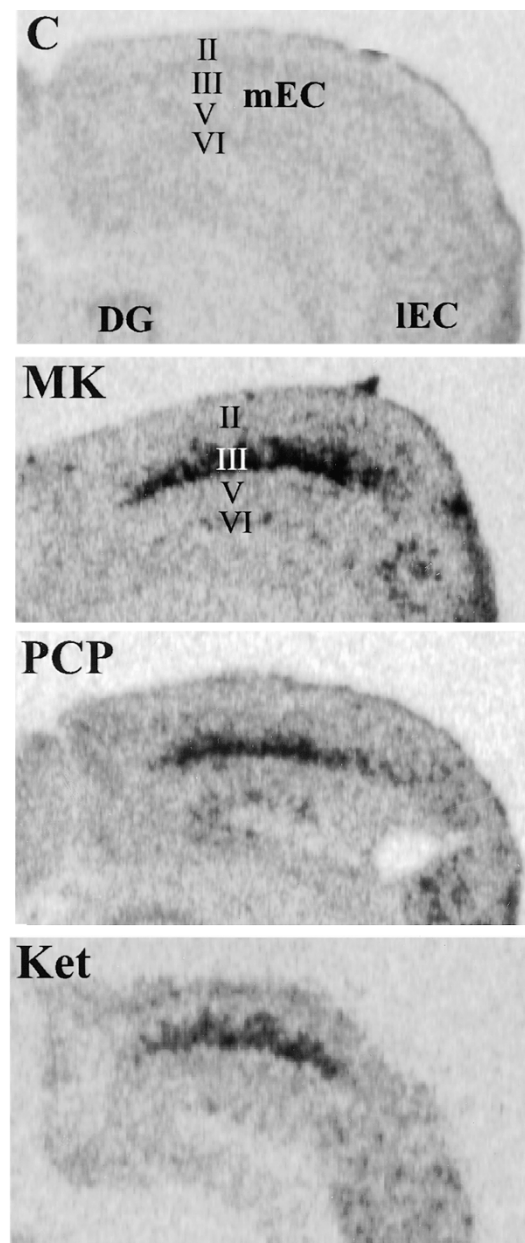

Figure 1.   Autoradiograms of in situ hybridization with c-fos antisense probe in horizontal sections of rat brain before (C) and 4 hours after administration of MK-801 (MK, 5 mg/kg), phencyclidine (PCP, 15 mg/kg) or ketamine (Ket, 50 mg/kg). (DG = dentate gyrus, lEC = lateral entorhinal cortex, mEC = medial entorhinal cortex. Roman numerals indicate layers of the entorhinal cortex. Magnification 15×.)
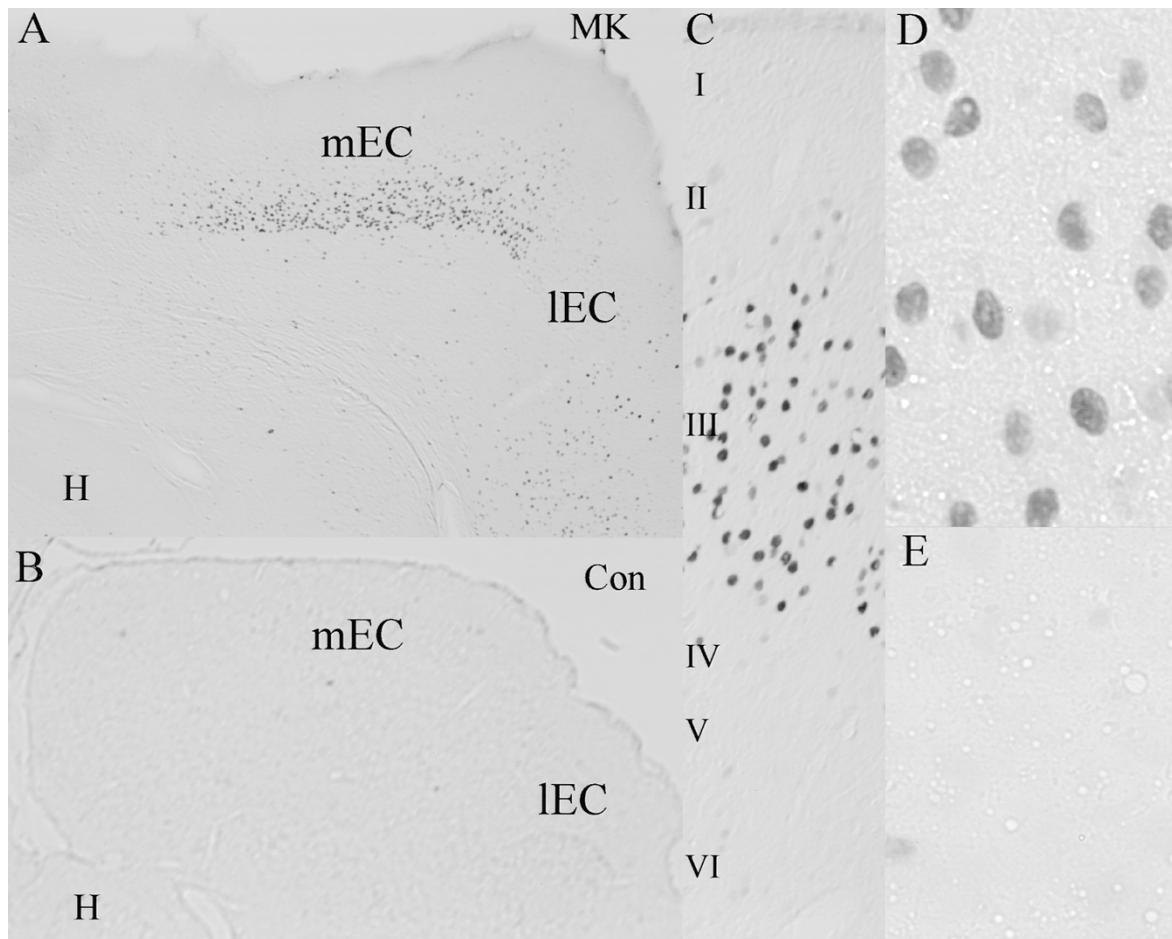
Figure 2.   c-fos immunoreactivity in horizontal sections of rat brain 6 hours after injection of MK-801 (5 mg/kg) (A, C, D) or a control brain (B, E). (A) c-fos immunoreactivity is mostly seen in the medial entorhinal cortex (mEC) and to much lesser extent, in the lateral entorhinal cortex (lEC). No immunoreactivity was seen in the hippocampus (H). (C) Section through layers (I–VI) of the mEC of a MK-801 treated rat. Positive neurons are concentrated in layer III. (D and E) High magnification of neurons in layer III of the mEC of a MK-801-treated rat (D) and a control rat (E).

Paxinos and Watson (1986)]. However, no induction of c-fos was observable in the most ventral portion of the medial EC (bregma −7.6 and more ventral to that). The region where MK-801 induces c-fos mRNA and protein corresponds to the region nominated caudal EC (cEC) by Amaral (Amaral and Witter 1995). In coronal sections cEC is located at the caudal pole of the forebrain and is easily missed, when only coronal sections are used in the analysis.

Induction of c-fos mRNA was dose-dependent (Figure 4A). A dose of 0.1 mg/kg, which did not produce any behavioral alteration (data not shown), already significantly increased c-fos mRNA levels. The increase in c-fos mRNA levels did not completely saturate even at the highest dose tested (5 mg/kg) which may reflect nonspecific effects of MK-801 on other receptor systems at these high concentrations. The half-maximal dose was 0.42 mg/kg (considering 5 mg/kg as a maximal dose). Induction of c-fos mRNA was detectable within

30 minutes after MK-801 injection (Figure 4B). One hour after the administration, a modest but significant c-fos mRNA increase was detectable. The induction of c-fos mRNA was at its highest 4 hours after the administration (577% over control at 4 hours). At 12 hours, mRNA had returned to close to baseline but remained slightly but significantly ($p < .05$) increased until 48 hours after the administration.

Antipsychotic drugs have been shown to inhibit NMDA-receptor induced vacuole formation in the retrosplenial cortex (Farber et al. 1995). Two antipsychotic drugs, clozapine and haloperidol significantly inhibited the c-fos induction induced by 5 mg/kg of MK-801 (Figure 5). In two of four rats, c-fos mRNA induction was essentially abolished by both clozapine and haloperidol, but residual induction was observed in another two rats in both groups. Interestingly, even in rats where c-fos mRNA induction by MK-801 was completely abolished by clozapine and haloperidol in the
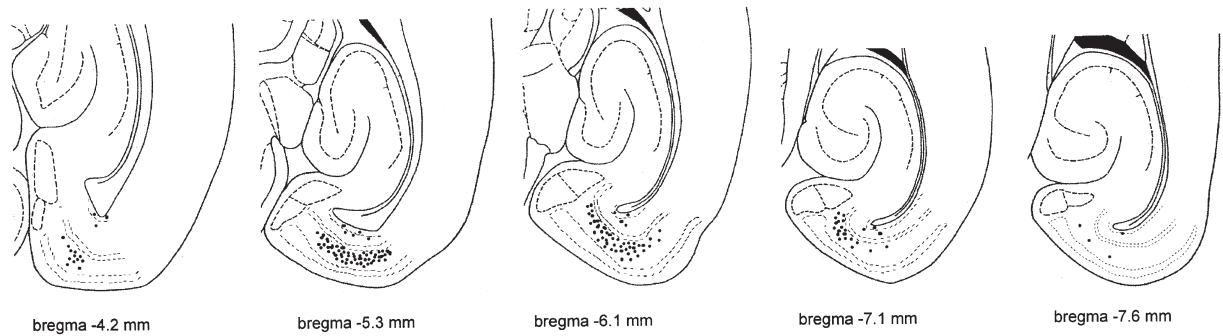
bregma -4.2 mm   bregma -5.3 mm   bregma -6.1 mm   bregma -7.1 mm   bregma -7.6 mm

Figure 3.   Distribution of c-fos mRNA positive neurons in the caudal entorhinal cortex from dorsal (left) toward ventral (right) sections. The section at bregma −7.6 and sections more ventral to that did not show significantly higher c-fos mRNA levels that control sections. Sections correspond to those in Paxinos and Watson (1986).

entorhinal cortex, cells expressing high amounts of c-fos mRNA were still observed in the central gray area, where MK-801 is known to induce c-fos and BDNF mRNA expression (Dragunow and Faull 1990; Castrén et al. 1993) (data not shown). In contrast, the antidepressant desipramine did not significantly influence MK-induced c-fos mRNA synthesis in the EC.

Effects of MK-801 and PCP in cultured entorhinal cortical neurons   Cultured primary neurons offer an experimental model that is much more easily manipulated than living animals. We therefore investigated whether the excitatory effects of PCP and MK-801 observed in vivo could be reproduced in cultured neurons dissected from the entorhinal cortex. Moderate doses of MK-801 (10 μM) did not produce any toxic effects in

cultured entorhinal cortical neurons after the exposure of 2 days (Figure 6A–C). In fact, the cells appeared healthier in the presence of a low dose of MK-801 (Figure 6B), which is to be expected from the blockade of the neurotoxic effects of endogenously released glutamate. However, high concentrations of both MK-801 and PCP were toxic to EC neurons. MK-801 at the concentration of 50 μM induced the appearance of intracellular vacuoles in neurons (Figure 6C) and at 100 μM of MK-801 neurons begun to lose their processes (Figure 6D). At still higher concentrations of MK-801 (200 μM, Figure 6E) and PCP (500 μM, Figure 6F) the cells lysed. This process was not immediate, but appeared gradually within the first 2 days of exposure.

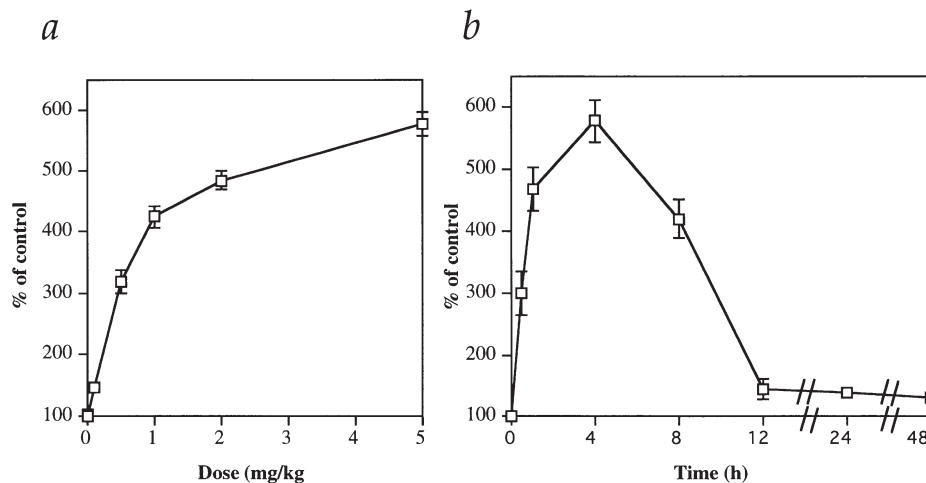Neurotoxic effects of MK-801 and PCP were dose-dependent (Figure 7). The effective doses of MK-801

Figure 4.   Effect of intraperitoneal MK-801 injection on c-fos mRNA expression in the entorhinal cortex. (A) Expression 4 hours after injections of different doses compared with saline-treated controls. Each point represents the mean and SEM of four animals (except 0.1 mg/kg treatment group, where $n = 2$). (B) Time course of expression after 5 mg/kg IP injection compared with saline-treated controls. The values at all the time points are significantly different from the control (time 0) at the level of $p < .05$ (ANOVA). Mean ± SEM, $n = 8$.
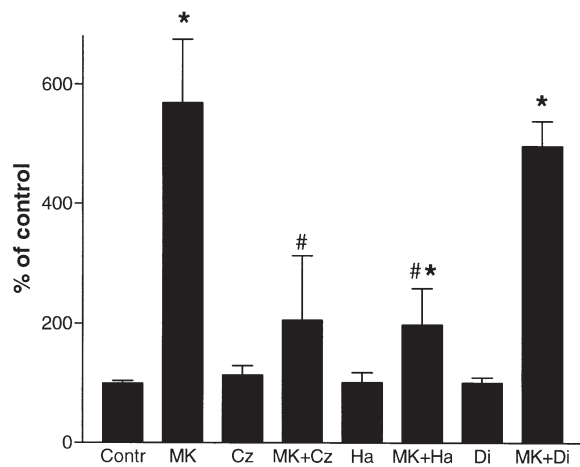
Figure 5. Effects of clozapine (Cz, 25 mg/kg), haloperidol (Ha, 2 mg/kg), and desipramine (Di, 15 mg/kg) on c-fos mRNA levels induced by MK-801 (MK, 5 mg/kg).*$p < .05$ against control, # $p < .05$ against MK-801 (ANOVA: Contr vs. MK, F = 0.0030, $p < .001$; Contr vs. Cz, F = 0.14, $p > .05$; Contr vs. Ha, F = 0.114, $p > .05$; Contr vs. Di, F = 0.45, $p > .05$; Contr vs. MK + Cz, F = 0.0029, $p < .001$; Contr vs. MK + Ha, F = .0091, $p < 0.01$; Contr vs. MK + Di, F = 0.020, $p < .01$; MK vs. MK + Cz, F = 0.093, $p < .05$; MK vs. MK + Ha, F = 0.13, $p < .05$; MK + Di, F = 0.33, $p > 0.05$). Mean ± SEM, $n = 5$.

were approximately half of that of PCP, which is consistent with the lower affinity of PCP to the NMDA receptor as compared to MK-801. Pentobarbital, which protects neurons in the RSC and EC from MK-801 and PCP-induced effects in vivo (Olney et al. 1991; Castrén et al. 1993), had a small but significant protective effect against a high dose of MK-801 (Figure 7).

Synthesis of brain-derived neurotrophic factor (BDNF) is strongly induced in both EC and RSC after MK-801 injection in vivo (Castrén et al. 1993; Hughes et al. 1993) and BDNF has been reported to be a survival factor for cortical neurons in vitro (Ghosh et al. 1994; Acheson et al. 1995). We therefore tested whether addition of BDNF could protect neurons against MK-801 and PCP-induced toxicity. As shown in Figure 7, addition of BDNF (50 ng/ml) was not able to rescue cultured EC cells from the toxic effects of MK-801.

We also tested the effect of antipsychotic drugs on the survival of entorhinal cortical neurons after the exposure to MK-801. We used haloperidol and chlorpromazine in these experiments, because clozapine could not be reliably dissolved into the culture medium. Haloperidol did not significantly influence the survival on neurons in the presence of either 50 μM (not shown) or 200 μM of MK-801 (Figure 7). Chlorpromazine also did not help to rescue neurons, on the contrary, it significantly reduced the survival of entorhinal cortical neurons in the presence of both 50 μM (not shown) and 200 μM MK-801 (Figure 7).

To test whether the neurotoxicity of MK-801 and PCP in cultured neurons could act as a valid model for excitatory effects observed in the limbic cortex in vivo, we investigated whether the expression of c-fos is increased in cultured EC neurons as it is in vivo. As shown in Table 1, neither MK-801 nor PCP increased c-fos expression in these neurons at any of the concentrations or time points investigated. On the contrary, there was a significant reduction in the c-fos expression at all time points except 1 hour after the lower MK-801 concentration (Table 1). This is most probably caused by the blockade of NMDA receptor-mediated elevation of c-fos in neurons by spontaneous synaptic activity. As expected, kainic acid (25 μM) produced a clear induction of c-fos immunoreactivity in these cultures within 1 hour (Table 1).

DISCUSSION

We have characterized excitatory effects of non-competitive NMDA receptor antagonists in the rat entorhinal cortex and in cultured neurons prepared from embryonic rat entorhinal cortex using c-fos induction as an indicator. We have observed a remarkably strong, dose-dependent and spatially restricted expression of c-fos mRNA and protein in layer III of the caudal subfield in the entorhinal cortex by all the three NMDA receptor antagonists tested: MK-801, phencyclidine, and ketamine.

Neurons in layer III of the EC play an important role in the highly organized information processing within the EC-hippocampus loop. Input from all sensory modalities is concentrated to neurons in the superficial layers (II and III) of the EC from where processed information is funneled to the hippocampus through two pathways (Jones 1993; Amaral and Witter 1995). Neurons in layer II send their projections through the perforant path to the dentate gyrus, which is the starting point of the so-called tri-synaptic loop through the hippocampus. Layer III neurons (which are affected by PCP) preferentially send their projections into the CA1 area and subiculum, and these projections are thought to be important in the control of the output from hippocampus back to deep layers of the EC (Amaral and Witter 1995). Sensory information that is processed in these two parallel EC-hippocampus loops is then distributed back to different parts of cortex and subcortical structures. We have previously shown that MK-801 at doses that were shown here to increase c-fos mRNA expression in the entorhinal cortex abolishes the characteristic long-lasting inhibition in mEC layer III cells which normally is produced by repetitive stimulation (Gloveli et al. 1997). This loss of inhibition may disrupt the organized information flow from entorhinal cortex
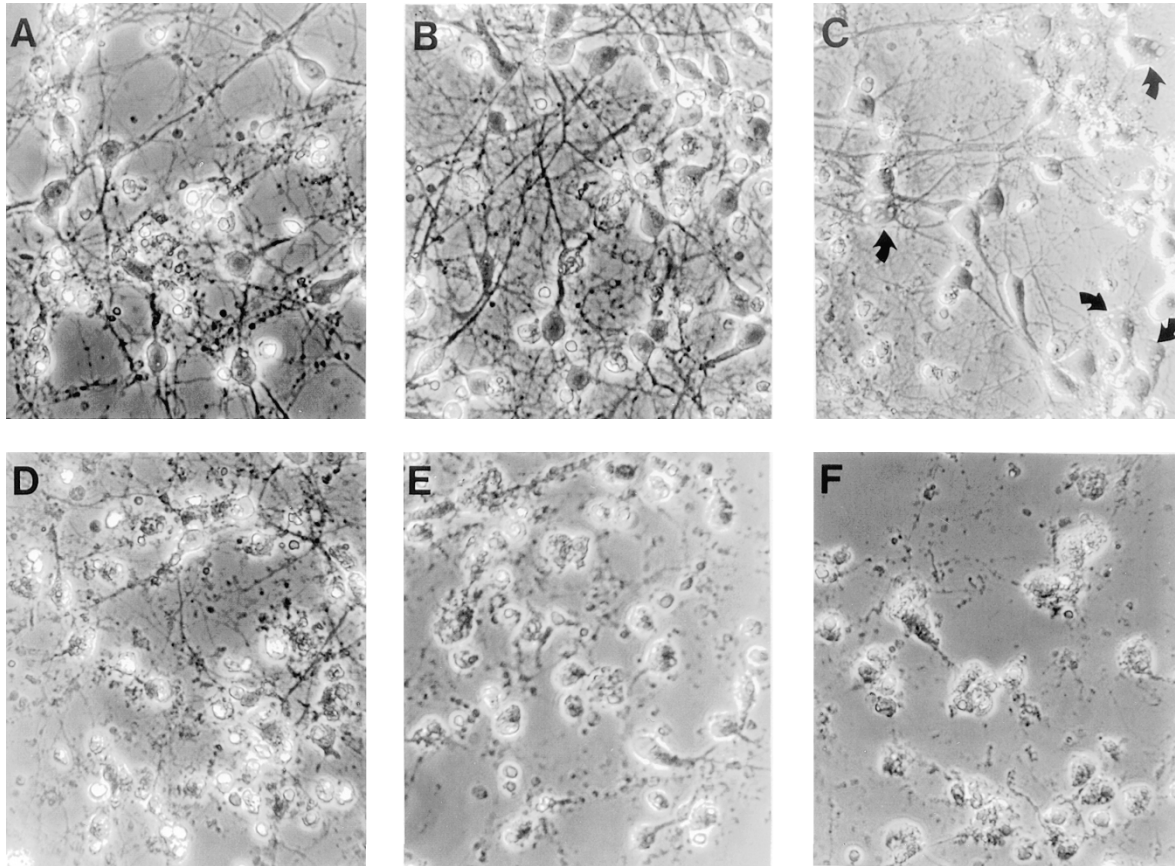
Figure 6. Cortical neurons from E17 embryonic rat entorhinal cortex cultured for 7 days in serum-free culture medium (A) or for 5 days in medium only and then for 2 additional days together with 10 μM MK-801 (B), 50 μM PCP (C), 100 μM MK-801 (D), 200 μM MK-801 (E), or 500 μM PCP (F). Arrows in C indicate neurons with intracellular vacuoles. Magnification 400×.

to hippocampus and back and may well produce behavioral disturbances.

Neurons in the caudal subdivision of the EC receive preferential input from the limbic system, in particular from the cingulate and retrosplenial cortex (Amaral and Witter 1995). This area is also a target of a heavy input from thalamus, in particular from the reuniens and central medial nuclei (Amaral and Witter 1995). Remarkably, precisely these cortical and thalamic areas display increased immediate early gene expression in response to NMDA receptor antagonists (Dragunow and Faull 1990; Castrén et al. 1993). This suggests that these brain areas may constitute a neuronal circuit, which responds by excitation to the administration of NMDA-receptor antagonists. It is also interesting to note that brain areas which are activated in schizophrenic patients experiencing hallucinations includes parahippocampal gyrus (which mostly consists of entorhinal cortex) and cingulate cortex, areas where MK-801 induce c-fos expression (Silbersweig et al. 1995). PET method used for these human studies do not, however, allow a cellular or even layer-specific identification of the affected cells.

The half-maximal dose needed for c-fos induction in the EC in vivo was similar to that described for neuronal vacuolization in the posterior cingulate cortex in male rats (Olney et al. 1989) and antipsychotic drugs inhibit both processes (Farber et al. 1993). These data suggest that a similar kind of mechanism may explain the induction of c-fos mRNA and protein in the EC and neuronal vacuolization in the retrosplenial cortex. However, neuronal vacuolization has not been observed in the EC and we did not find abnormally high numbers of apoptotic cells (as detected by TUNEL staining, data not shown) in this brain area at least 24 hours after the drug administration. It appears that neurons in the retrosplenial cortex are more sensitive to the toxic effects of NMDA receptor antagonists. This is consistent with the observations that repeated administration of MK-801 (Horvath et al. 1997) or the combination of PCP with pilocarpine (Corso et al. 1997) produces cell death not only in the RSC but also in the EC.

We have sought to develop a cell culture system which would replicate the excitatory effects of NMDA receptor antagonists observed in vivo to be used as an
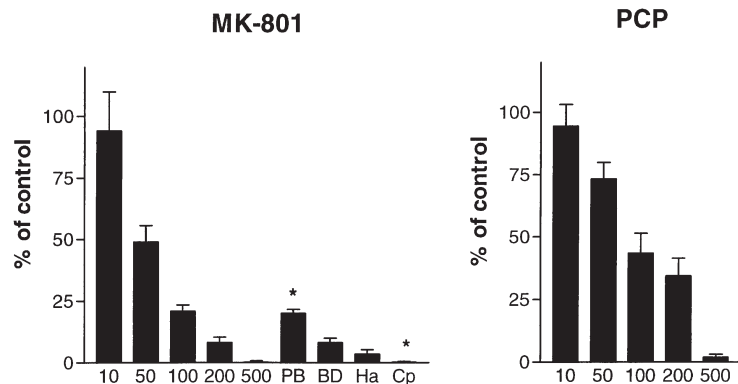
Figure 7.   Survival of neurons from embryonic entorhinal cortex in the presence of different concentrations of MK-801 (left) or phencyclidine (PCP, right). Neurons were cultured for 5 days in control medium and then treated for 2 days with indicated concentrations of drugs and viable cells were counted under phase-contrast microscopy. (PB = phenobarbital, 200 μM, BD = BDNF, 50 ng/ml, Ha = haloperidol (1 μM), Cp = chlorpromazine (1 μM). * significantly different from MK-801 200 μM. $p < .05$, ANOVA.)

in vitro model in antipsychotic drug development. Our data demonstrate that cultured neurons from embryonic rat EC display many, but not all neurotoxic features that have been reported to be induced by PCP and MK-801 in the RSC. Thus, PCP and MK-801 are neurotoxic to EC neurons at high concentrations. This toxicity could be at least partially prevented by pentobarbital, which blocks PCP neurotoxicity in the RSC in vivo (Olney et al. 1991). BDNF, which is strongly increased both in the RSC and in the EC by MK-801 (Castrén et al. 1993; Hughes et al. 1993), did not rescue EC neurons from PCP toxicity in vitro and neither did antipsychotic drugs haloperidol and chlorpromazine. On the other hand, doses needed to kill neurons were for both drugs much higher than those needed to block NMDA receptors. This suggests that neurotoxic effects in this culture system may not solely be mediated through the blockade of NMDA receptors but may reflect interaction with other receptors or channels. Indeed, evidence exist that neurotoxic effects of PCP in embryonic human cortical neurons may be mediated through blockade of potassium channels (Mattson et al. 1992).

The fact that c-fos immunoreactivity is not increased (it is rather decreased) in EC cells in vitro as it is in vivo, together with the high concentrations of both PCP and MK-801 needed for the toxicity in vitro, suggest that the excitatory reaction observed in the limbic cortex by PCP and MK-801 in vivo is probably at least partially produced through a different mechanism than the in vitro toxicity. This is further supported by the electrophysiological observation that indicates that reduced inhibition of layer III neurons in the entorhinal cortex of rats treated with MK-801 in vivo could not be observed in brain slice from a normal rat after bath application of MK-801 (Gloveli et al. 1997). Based on their pharmacological data, Olney and coworkers have suggested a neuronal circuit model for the neurotoxic effects of MK-801 and PCP in RSC (Olney et al. 1991; Olney and Farber 1995a). Elements required for the development of such neuronal circuits are not present in our dissociated EC neuron model. Furthermore, it has been demonstrated that neurotoxic effects of MK-801 and PCP can only be observed in adult rats older that 4 weeks (Farber et al. 1995). For neuronal cultures it is necessary to use neurons from embryonic brain and it is questionable whether maturation steps necessary for the development of the selective vulnerability in the limbic cortex takes place in culture.

In conclusion, although the in vitro neuronal toxicity of NMDA receptor antagonists is an interesting phe-

Table 1.   c-fos Immunoreactive Cells after MK-801 or PCP Treatment (% of Control)

Time	MK 2 μM	MK 80 μM	PCP 10 μM	PCP 200 μM	KA 25 μM
1h	90 $\pm$ 29	60 $\pm$ 19%*	60 $\pm$ 37	62 $\pm$ 21	414 $\pm$ 55%*
2h	59 $\pm$ 15%*	16 $\pm$ 8%*	14 $\pm$ 10%*	20 $\pm$ 10%*	
4h	24 $\pm$ 12%*	35 $\pm$ 30%*	15 $\pm$ 16%*	23 $\pm$ 16%*	
8h	16 $\pm$ 12%*	30 $\pm$ 10%*	6 $\pm$ 5%*	7 $\pm$ 6%*	
24h	12 $\pm$ 10%*	32 $\pm$ 12%*	16 $\pm$ 10%*	15 $\pm$ 10%*	

$n = 5$ for each value
*$p < .05$ (ANOVA).

nomenon that might be useful in the development of less toxic antagonists, it appears that cultured entorhinal neurons do not serve as a reliable in vitro model of the excitatory effects of NMDA receptor antagonists as they are observed in vivo.

The remarkable and highly localized excitatory effects induced by NMDA antagonists in the limbic cortex may underlie the psychotomimetic side effects of these drugs (Olney and Farber 1995a, 1995b). These effects may mimic disturbances in neuronal connectivity which produce psychotic symptoms in schizophrenics. Thus, these excitatory alterations could be used to delineate the neuronal circuits that, when functioning abnormally, produce psychotic behavior. Because of the important role of the EC in the processing of sensory input (Jones 1993) and disturbances in the neuronal organization and activity repeatedly observed in the EC neurons in schizophrenic brains (Falkai et al. 1988; Arnold et al. 1991; Bogerts 1993; Jakob and Beckmann 1994; Silbersweig et al. 1995), caudal EC is a strong candidate for the brain area that is affected in schizophrenia. The relatively large number of affected neurons in the cEC and the wealth of information concerning the connectivity and physiology of the entorhinal-hippocampal system render this brain area a good candidate for a model system. Further studies that characterize the development and connectivity of neurons in layer III of the EC as well as those analyzing biochemical and molecular events taking place in these neurons in response to NMDA receptor antagonists may help to understand the cellular pathology underlying schizophrenic psychosis.

ACKNOWLEDGMENTS

REFERENCES

Acheson A, Conover JC, Fandl JP, DeChiara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM (1995): A BDNF autocrine loop in adult sensory neurons prevents cell death. Nature 374:450–453

Amaral DG, Witter MP (1995): Hippocampal formation. In Paxinos G (ed), The Rat Nervous System. Sydney, Academic Press, pp 443–493

Arnold SE, Lee VM, Gur RE, Trojanowski JQ (1991): Abnormal expression of two microtubule-associated proteins (MAP2 and MAP5) in specific subfields of the hippocampal formation in schizophrenia. Proc Natl Acad Sci USA 88:10850–10854

Bogerts B (1993): Recent advances in the neuropathology of schizophrenia. Schizophrenia Bull 19:431–445

Castrén E, Berzaghi MP, Lindholm D, Thoenen H (1993): Differential effects of MK-801 on the brain-derived neurotrophic factor mRNA levels in different regions of rat brain. Exp Neurol 122:244–252

Corso TD, Sesma MA, Tenkova TI, Der TC, Wozniak DF, Farber NB, Olney JW (1997): Multifocal brain damage induced by phencyclidine is augmented by pilocarpine. Brain Res 752:1–14

Dragunow M, Faull RLM (1990): MK-801 induces c-fos protein in thalamic and neocortical neurons of rat brain. Neurosci Lett 113:144–150

Falkai P, Bogerts B, Rozumek M (1988): Limbic pathology in schizophrenia: The entorhinal region-a morphometric study. Biol Psychiatry 24:515–521

Farber NB, Wozniak DF, Price MT, Labruyere J, Huss J, St. Peter H, Olney JW (1995): Age-specific neurotoxicity in the rat associated with NMDA receptor blockade: Potential relevance to schizophrenia? Biol Psychiatry 38:788–796

Farber NF, Price MT, Labruyere J, Nemnich J, St. Peter H, Wozniak DF, Olney JW (1993): Antipsychotic drugs block phencyclidine receptor-mediated neurotoxicity. Biol Psychiatry 34:119–121

Ghosh A, Carnahan J, Greenberg ME (1994): Requirement for BDNF in activity-dependent survival of cortical neurons. Science 263:1618–1623

Gloveli T, Iserhot C, Schmitz D, Castrén E, Behr J, Heinemann U (1997): Systemic administration of the phencyclidine compound MK-801 affects stimulus-induced field potentials selectively in layer III of rat medial entorhinal cortex. Neurosci Lett 221:93–96

Horvath ZC, Czopf J, Buzsaki G (1997): MK-801-induced neuronal damage in rats. Brain Res 753:181–195

Hughes P, Dragunow M, Beilharz E, Lawlor P, Gluckman P (1993): MK801 induces immediate-early gene proteins and BDNF mRNA in rat cerebrocortical neurones. NeuroReport 4:183–186

Jakob H, Beckmann H (1994): Circumscribed malformation and nerve cell alterations in the entorhinal cortex of schizophrenics: Pathogenetic and clinical aspects. J Neural Transm Gen Sect 98:83–106

Javitt DC, Zukin SR (1991): Recent advances in the phencyclidine model of schizophrenia. Am J Psychiatry 148:1301–1308

Johnson KM, Jones SM (1990): Neuropharmacology of phencyclidine: Basic mechanisms and therapeutic potential. Annu Rev Pharmacol Toxicol 30:707–750

Jones RSG (1993): Entorhinal-hippocampal connections: A speculative view of their function. Trends Neurosci 16:58–64

Krystal JH, Karper LP, Seibyl JP, Freeman GK, Delaney R, Bremner JD, Heninger GR, Bowers MB, Charney DS (1994): Subanesthetic effects of the noncompetitive NMDA antagonist, ketamine, in humans: Psychotomimetic, Perceptual, Cognitive, and Neuroendocrine Responses. Arch Gen Psychiatry 51:199–214

Lindholm D, Hengerer B, Castrén E (1993): In vitro and in vivo methods for evaluating actions of cytokines on

nerve growth factor production in central nervous system. In DeSouza E (ed), Neurobiology of Cytokines, Methods in Neurosciences, Vol. 17 San Diego, Academic Press, pp 37–60

Mattson MP, Rychlik B, Cheng B (1992): Degenerative and axon outgrowth-altering effects of phencyclidine in human fetal cerebral cortical cells. Neuropharmacology 31:279–291

Morgan JI, Curran T (1991): Stimulus-transcription coupling in the nervous system: Involvement of the inducible proto-oncogenes fos and jun. Annu Rev Neurosci 14:421–451

Näkki R, Koistinaho J, Sharp FR, Sagar SM (1995): Cerebellar toxicity of phencyclidine. J Neurosci 15:2097–2108

Näkki R, Sharp FR, Sagar SM, Honkaniemi J (1996): Effects of phencyclidine on immediate early gene expression in the brain. J Neurosci Res 45:13–27

Olney JW, Farber NB (1995a): Glutamate receptor dysfunction and schizophrenia. Arch Gen Psychiatry 52:998–1007

Olney JW, Farber NB (1995b): NMDA antagonists as neuro-therapeutic drugs, psychotogens, neurotoxins, and research tools for studying schizophrenia. Neuropsychopharmacology 13:335–345

Olney JW, Labruyere J, Price MT (1989): Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. Science 244:1360–1362

Olney JW, Labruyere J, Wang G, Wozniak DF, Price MT, Sesma MA (1991): NMDA antagonist neurotoxicity: Mechanism and prevention. Science 254:1515–1518

Paxinos G, Watson C (1986): The Rat Brain in Stereotaxic Coordinates. 2nd ed. Sydney, Academic Press

Roberts GW (1990): Schizophrenia: The cellular biology of a functional psychosis. Trends Neurosci 13:207–211

Sharp FR, Butman M, Koistinaho J, Aardalen K, Näkki R, Massa SM, Swanson RA, Sagar SM (1994): Phencyclidine induction of the hsp70 stress gene in injured pyramidal neurons is mediated via multiple receptors and voltage gated calcium channels. Neuroscience 62:1079–1092

Sharp FR, Jasper P, Hall J, Noble L, Sagar SM (1991): MK-801 and ketamine induce heat shock protein HSP72 in injured neurons in posterior cingulate and retrosplenial cortex. Ann Neurol 30:801–809

Silbersweig DA, Stern E, Frith C, Cahill C, Holmes A, Groon-toonk S, Seaward J, McKenna P, Chua SE, Schnorr L, Jones T, Frackowiak RSJ (1995): A functional neuroanatomy of hallucinations in schizophrenia. Nature 378:176–179