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Learning deficits induced by chronic intraventricular infusion of quinolinic acid – protection by MK-801 and memantine

Marcin Misztal, Tadeusz Frankiewicz, Chris G. Parsons, Wojciech Danysz *

Department of Pharmacology, Merz + Co., Eckenheimer Landstrasse 100-104, 60318 Frankfurt / M, Germany

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Abstract

The NMDA receptor agonist quinolinic acid (9 mM) was infused i.c.v. via ALZET osmotic minipumps for 2 weeks. This treatment produced a persistent, short-term memory deficit in the T-maze. Autoradiography revealed a decrease in the density of choline uptake sites in the hippocampus. Parallel s.c. infusion by another minipump of the uncompetitive NMDA receptor antagonist memantine (1-amino-3,5-dimethyladamantane, 20 mg/kg per day) or (+)-5-methyl-10,11-dihydro-5*H*-dibenzocyclohepten-5,10-imine maleate ((+)-MK-801, 0.31 mg/kg day) prevented the learning deterioration induced by quinolinic acid. The treatment with memantine resulted in steady-state serum levels of 1.2 μM which, based on in vitro data, should assure inhibition of NMDA receptors and are similar to levels seen in the serum of demented patients treated with this agent. In naive animals this treatment had no effect on either learning or on ex vivo induction of long-term potentiation, indicating that under chronic conditions it is possible to obtain neuroprotective effects with NMDA receptor antagonists without negative effects on memory processes. This contrasts to some acute insults (e.g. ischaemia) where high doses of NMDA receptor antagonists that produce side effects are required.

Keywords: Quinolinic acid, i.c.v. infusion; Learning; Long-term potentiation; Memantine; MK-801

1. Introduction

Recently the excitotoxic hypothesis of chronic neurodegenerative diseases has attracted considerable attention, resulting in a number of papers addressing this issue (Greenamyre et al., 1988; Meldrum and Garthwaite, 1990; Beal, 1992; Lees, 1993; Turski and Turski, 1993; Lipton and Rosenberg, 1994). The potential role of glutamate (or other excitotoxins) in chronic, progressive neurodegeneration is interesting both in terms of basic science and also as a starting point for the search for neuroprotective therapies that might potentially slow down the progression of a given disorder. It is rather clear that glutamate cannot be judged to be the major aetiological factor in such conditions, however its potential role in the pathomechanism is hard to neglect, based on its excitotoxic potential (Meldrum and Garthwaite, 1990; Beal, 1992; Lees, 1993). Indeed, glutamate antagonists have been shown to be effective protectants in numerous acute models of ischaemia (Scatton, 1994). In this respect NMDA receptor antagonists have been studied the most, mainly due to their favourable pharmacokinetic properties. Unfortunately, the doses of NMDA receptor antagonists used in such acute models are well documented to produce numerous side effects such as memory deficits (Morris et al., 1986; Collingridge and Singer, 1990; Scatton, 1994; Danysz et al., 1995). However, it might be expected that neuroprotective activity in chronic neurodegenerative diseases can be achieved with lower doses of NMDA receptor antagonists, which would result in a better therapeutic profile.

To mimic chronic neurodegenerative conditions, models are needed that have features of progressive, persistent alterations induced by chronic insult (Danysz and Schmidt, 1994). In fact, recently several attempts have been made to address this issue properly. These attempts can be divided into three categories, those based on lowering brain blood supply i.e. chronic hypoperfusion (De la Torre et al., 1992; Ni et al., 1994),

* Corresponding author. Tel.: (+49) 69-150-35-64; fax: (+49) 69-596-21-50.

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impairing the respiratory chain in mitochondria i.e. by 3-nitropropionic acid (Beal et al., 1993) and infusion of excitotoxins (e.g. quinolinic acid) directly into the brain (Yamada et al., 1990; Susel et al., 1991; Vezzani et al., 1991; Bazzett et al., 1994). Their common feature is that continuous application of the insult produces progressive biochemical, histological and behavioural changes. In some of these models a progressive learning deficit has been observed. De la Torre et al. (1993) observed impairment of spatial learning in the Morris maze of rats within 3 weeks after permanent ligation of both common carotid arteries combined with ligation of the subclavian artery. A similar deficit was seen in the radial maze in rats subjected only to bilateral common carotid artery occlusion (Ni et al., 1994). Using direct i.c.v. infusion of the NMDA receptor agonist quinolinic acid, Yamada et al. (1990) also observed a progressive (over 3 weeks) deficit in cholinergic markers in the cortex and hippocampus. This treatment also produced an active avoidance deficit (Yamada et al., 1991). In all cases a similarity to some aspects of excitotoxicity possibly occurring in neurodegenerative dementia has been suggested by the authors.

Although the above models are well characterized, up to now none of the potential neuroprotective agents has been tested. In such tests three requirements should be fulfilled to approach therapeutic relevance. First, steady-state (or 'pseudo steady-state') levels of a given agent in the serum/brain should be assured; second this level should match the clinically relevant range; third a behavioural measure should be used to verify neuroprotective activity. To achieve these goals, continuous systemic delivery of neuroprotective agents by ALZET osmotic minipumps can be used and the drug concentration should be adjusted on the basis of serum levels. In this study the neuroprotective activity of the NMDA receptor channel blockers (+)-5-methyl-10,11-dihydro-5*H*-dibenzocyclohepten-5,10-imine maleate ((+)-MK-801), the most selective agent in this class (Lodge and Johnson, 1990), and 1-amino-3,5-dimethyladamantane (memantine), a clinically used agent for the treatment of dementia (Görtelmeyer et al., 1993; Pantev et al., 1993; Kornhuber et al., 1994), were tested.

To address the question whether chronically administered NMDA receptor antagonists interfere with learning at low doses that are still neuroprotective, we studied the effects of chronic infusion of MK-801 and memantine on learning deficits induced by chronic i.c.v. infusion of quinolinic acid (according to Yamada et al., 1990). In naive animals the effect of this treatment on learning and long-term potentiation was tested as a verification of possible negative changes on learning processes per se. The results have been previously presented in abstract form (Danysz et al., 1994b; Misztal et al., 1994).

2. Materials and methods

2.1. Subjects

Male Sprague-Dawley rats weighing 200–220 g or 160–180 g at the beginning of behavioural or long-term potentiation experiments, respectively, were housed in polyethylene cages (four to five per cage) initially with free access to food and water. After 3 days of adaptation, food intake was restricted to 15 g/animal per day to maintain their weight and reach appropriate levels of motivation. Room conditions were kept constant at 20°C, 50% relative humidity and with a 12/12-h dark/light cycle with light on at 06:00 h.

2.2. Chemicals

The following agents were used in the course of the present study; quinolinic acid (Sigma Chemicals, St. Louis, Mo., USA); 1-amino-3,5-dimethyladamantane hydrochloride (memantine, Merz + Co., Frankfurt/M, Germany) and (+)-5-methyl-10,11-dihydro-5*H*-dibenzocyclohepten-5,10-imine maleate (MK-801, RBI, USA). All substances were dissolved in saline and pH was adjusted to 7.4 ± 0.1

2.3. Surgical procedure

Animals were fixed in the stereotaxic frame and the skull was exposed under Hypnorm anaesthesia (0.04 ml/100 g i.m.). Two holes were made, one for a steel cannula (AP = −1 mm, L = 1.5 mm relative to bregma) and the other for a fixing screw. An Alzet Brain Infusion Kit was used as a cannula and tubing for connection with the Alzet 2002 osmotic mini-pump. Cannulae were placed in position reaching 3.5–4.0 mm below skull level and fixed with dental cement. Pumps containing 9 mM quinolinic acid were implanted s.c. on the back of the animal and the wound was closed with 9 mm autoclips. MK-801- and memantine-treated groups underwent the same procedure but additionally received an implant of Alzet 2ML2 (memantine, 20 mg/kg per day) or 2002 (MK-801, 0.031 mg/kg per day) pumps delivering the drug s.c. Prior to implantation, filled pumps were placed in saline for 24 h in an incubator at 37°C. After 14 days the pumps were removed, and the tubing was sealed and left in place together with the cannulae. The test was started 3 days after removal of the pump.

To test the action of drugs in naive animals on learning and long-term potentiation, only pumps delivering the NMDA receptor antagonists were implanted s.c. and testing started 7 or 14 days after implantation respectively.

2.4. T-maze testing

The T-maze consisted of three identical arms (68 × 17 cm L × W, with 36 cm high walls) made of plastic-covered wood. Small glass containers for food pellets were placed in the end of the target arms.

After initial handling (2 days, 5 min per animal) rats were habituated to the apparatus for 2 days. During the first day animals were placed in the T-maze in groups of five for 5 min. During this time rats were allowed to move freely within the whole labyrinth. The glass containers contained five to seven food pellets per rat. During habituation each rat was removed from the T-maze and placed back in it 3–4 times. On the second day the procedure was the same except only one rat at a time was placed in the labyrinth. For 5–6 further days pre-training took place. Each rat was placed at the beginning of the starting arm and was supposed to enter one of the target arms within 30 s. If it failed to do so, the animal was removed from the maze for 15 s and then placed again at the beginning of the starting arm. As the rat entered the target arm it was allowed to reach the glass container and eat the pellet. Immediately afterwards the rat was moved to the beginning of the starting arm. During the first trial food pellets were placed in both containers (one per container). During following trials the pellet was placed in the arm not chosen in the previous trial. In case of a wrong choice (second consecutive entry to the same arm) the rat did not receive the pellet, an error was scored and the rat was moved to the starting arm. After animals reached a level of 60–70% of correct choices, pre-training was terminated. Animals were divided into groups such that the average of errors and S.D. were as similar as possible. Then animals underwent surgical procedures and after 8–17 days the test began. Tests were carried out for 5 days. Every day each animal received 11 trials and up to 10 errors could be scored (during the first trial any entry was a correct choice).

2.5. Receptor autoradiography

1 day after behavioural experiments were completed, the rats were decapitated and the brains were removed and rapidly frozen. Frozen brains were cut into 20 μm slices and placed on gelatin-coated glass slides. Then the slides were left at room temperature for 60 min and then placed in a deep-freezer (−72°C) until incubation (within 1 week). Incubation lasted 60 min in 50 mM Tris-HCl (pH 7.4) containing 300 mM NaCl and 20 nM [^{3}H]hemicholinium-3 (132.8 Ci/mmol, Du Pont NEN, USA). For unspecific binding 10 μM hemicholinium-3 (RBI, USA) was used. After incubation the slides were washed 6 times in Tris-HCl buffer (60 s each) and finally in distilled water. Slides were then dried under a stream of cold air. 24 h after incubation the slides and standard microscales ([^{3}H]Microscales, Amersham, UK) were placed in roentgen cassettes and covered with Hyperfilm (Amersham). Films were developed after 21 days of exposure.

2.6. Induction of the long-term potentiation

Chloroform-anaesthetised rats were decapitated on the 14th day of memantine infusion and the brains were removed rapidly and immediately cooled down (2–4°C). Transverse hippocampal slices (400 μm thick) were cut (FTB Vibrocut) and stored in artificial cerebro-spinal fluid (ACSF) containing (mM) NaCl 124, KCl 3, $NaHCO_3$ 26, NaH_2PO_4 1.2, $CaCl_2$ 2, $MgSO_4$ 1, D-glucose 10, bubbled with 95%O_2-5%CO_2 at room temperature, for at least 2 h. Slices from memantine-treated rats were kept in ACSF containing 1 μM memantine, through all the experimental steps. This concentration seems most relevant based on clinical studies (see Discussion).

For recording, slices were placed on a nylon mesh in an interface chamber (BSC-HT, Medical Systems) at 33°C in an oxygenated-enriched (95% O_2-5% CO_2) humidified atmosphere and perfused at a rate of 0.8ml/min with ACSF. After at least 30 min of incubation in the recording chamber, a glass recording electrode (1.5–3 MΩ, filled with ACSF) was positioned in the dendritic layer of CA1 to record extracellular field excitatory post-synaptic potentials (field EPSP). A bipolar stimulating electrode was placed between CA3 and CA1 region to activate the Schaffer-commissural fibers.

Extracellular recordings were made in response to constant voltage (10–20 V, 20 μs) single shock stimulation once every 15 s (0.066 Hz). Stimulus intensities were adjusted to evoke EPSPs of about half-maximal amplitude. To evoke long-term potentiation, tetanic stimulation (100 Hz, 1 s, 20 μs pulse width) was used.

Field potentials were digitized and stored for off-line analysis. The slope of the rising phase of the field EPSP (mV/ms) was measured between 20–80% of peak amplitude. Results were normalized and expressed as a means (±S.E.M.).

2.7. Measuring memantine concentrations

Rats were anaesthetised with pentobarbital (60 mg/kg, i.p.) and then blood was collected by heart punction and the brains were removed and frozen. The blood was then centrifuged at 4000 rpm. The supernatant was separated and frozen at −20°C until analysis. Brains (0.5 g of tissue) were treated with 2 ml of 2.5 M H_2SO_4 and 100 μl of internal standard (amantadine) at 90°C for 60 min. Extractions were then the

same for brain and serum samples. 1 ml of serum was pipetted into a culture tube. Then 1 ml of 2 M hydrochloric acid and 2 ml of internal standard (amantadine hydrochloride) was added. The sample was treated at 70°C for 15 min. After cooling to room temperature, 0.6 ml hexane and 0.6 ml 10 M NaOH were added. This mixture was extracted on a cooling mixer for 30 min and afterwards the organic phase was transferred into a GC-vial. The samples were then processed by a gas-chromatography system (5970/5971 Hewlett Packard) coupled to a mass selective detector. The analytical column (Restek Stabilwax DB L = 30 m, ID = 0.25 mm) was used with an injection mode splitless 1–3 μl and gas 10 psi helium 1 ml/min. The injection temperature was 220°C and the detection temperature was 240°C. The ionization mode was positive electrical ionization.

2.8. Statistical analysis

The results are expressed as means ± S.E.M. and were analysed by two-way repetitive measures ANOVA (time as repeated factor) for behavioural data or one way ANOVA for autoradiography data. If a significant effect was obtained with ANOVA, the Student-Newman-Keuls test for comparison between groups for the whole experiment duration was used. Additionally in case of behavioural studies one-way ANOVA followed by the Dunnett test was used to test significance on a particular day. The analysis was performed with SigmaStat software (Jandel Scientific) at $P < 0.05$ level of significance.

3. Results

3.1. Effect of NMDA receptor antagonists infusion on quinolinic acid-induced deficit

The i.c.v. infusion of quinolinic acid produced a clear-cut deficit of reinforced alternation in the T-maze 3–8 days after the termination of the infusion, as indicated by analysis of the whole study and on a particular day (Figs. 1 and 2). Parallel s.c. infusion of MK-801 (0.31 mg/kg per day) abolished the learning impairment induced by quinolinic acid (Fig. 1). Similarly, s.c. infusion of memantine (20 mg/kg per day) significantly prevented the development of this deficit (Fig. 2). There were no differences between control animals and the group treated with both memantine and quinolinic acid. At the dose used memantine reached a serum concentration of 1.2 μM, within 24 h after the start of infusion (Fig. 3). In the brain homogenates a concentration of 40 μmol/kg tissue was achieved (Fig. 3).

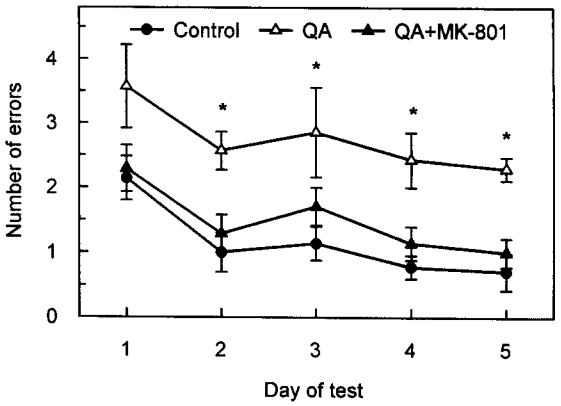

Fig. 1. S.c. infusion of MK-801 (0.31 mg/kg per day) prevents the development of the T-maze alternation deficit induced by chronic i.c.v. infusion of quinolinic acid (QA, 9 mM). Both agents were infused for 14 days and the test was started 3 days after termination of infusion. Values are means ± S.E.M. $^*P < 0.05$ as compared to control and QA + MK-801 treated groups (Dunnett test). $n = 8$.

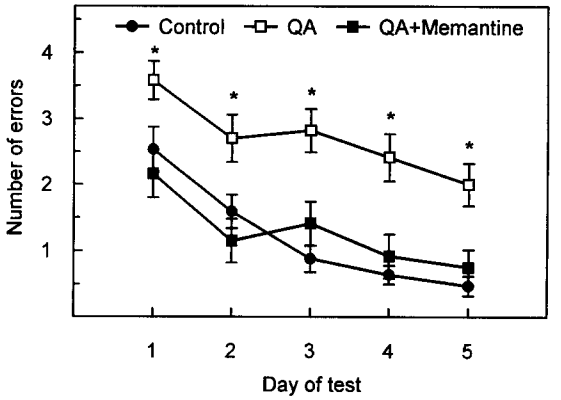

Fig. 2. S.c. infusion of memantine (20 mg/kg per day) prevents the development of the T-maze alternation deficit induced by chronic i.c.v. infusion of quinolinic acid (QA, 9 mM). Both agents were infused for 14 days and the test was started 3 days after termination of infusion. Values are means ± S.E.M.. $^*P < 0.05$ as compared to control and QA + memantine treated groups (Dunnett test). $n = 17$ for control and QA group and $n = 12$ for memantine + QA group.

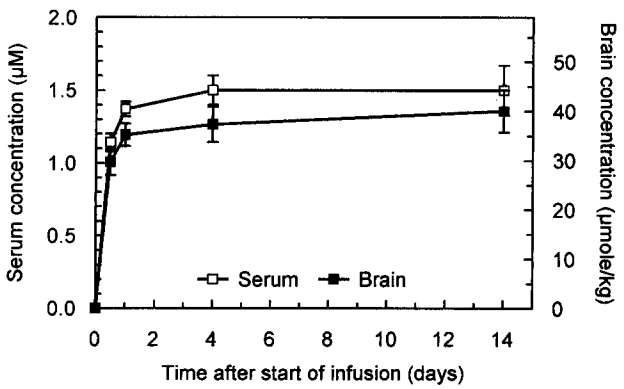

Fig. 3. Plasma and brain concentrations of memantine during s.c. infusion at a rate of 20 mg/kg per day. Results are means ± S.E.M.. $n = 4$.

Table 1
Effect of i.c.v infusion of quinolinic acid (9 mM) for 2 weeks alone or accompanied by parallel s.c. infusion of memantine (20 mg/kg per day) on [^{3}H]hemicholinium-3 binding in CA1 and CA3,4 hippocampal regions

Group	CA1	CA3,4
Control (5)	74.5 ± 2.33[a]	74.5 ± 2.49 [a]
Quinolinic acid (7)	40.6 ± 1.66	46.7 ± 2.64
Quinolinic acid + memantine (6)	78.3 ± 2.41 [a]	76.8 ± 1.36 [a]

Results are expressed as means ± S.E.M. μmol/mg tissue specific binding, as evidenced by autoradiography. Values in parentheses denote the number of animals per group. [a] $P < 0.05$ as compared to the group treated with quinolinic acid only (Student-Newman-Keuls test).

3.2. Effect of memantine treatment on quinolinic acid-induced decrease in [^{3}H]hemicholinium-3 binding

The infusion of quinolinic acid produced a significant decrease in [^{3}H]hemicholinium-3 binding in the hippocampal region (CA1, CA3,4), (Table 1). Memantine prevented this decrease, as evidenced by a significant difference between the quinolinic acid group and animals treated with quinolinic acid accompanied by s.c. infusion of memantine (Table 1).

3.3. Effect of NMDA receptor antagonist infusion on learning in naive rats

The infusion of MK-801 as well as memantine alone at the doses used (0.31 and 20 mg/kg per day respectively) did not affect alternation learning in the T-maze (Fig. 4). No behavioural changes were observed during the whole course of infusion.

3.4. Induction of the long-term potentiation

High-frequency stimulation induced stable NMDA receptor-dependent long-term potentiation (> 150 min)

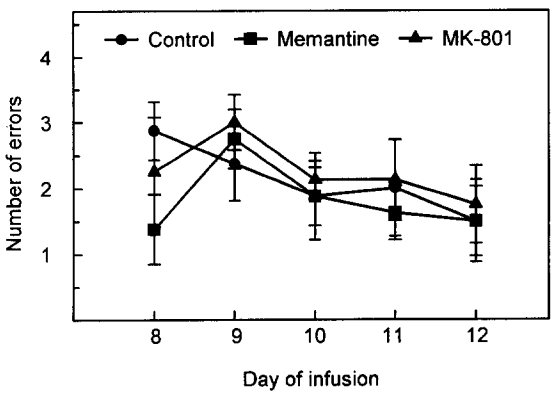

Fig. 4. Effect of s.c. infusion of MK-801 (0.31 mg/kg per day) and memantine (20 mg/kg per day) on T-maze alternation. The test started on the 8th day of infusion. Values are means ± S.E.M. $n = 8$.

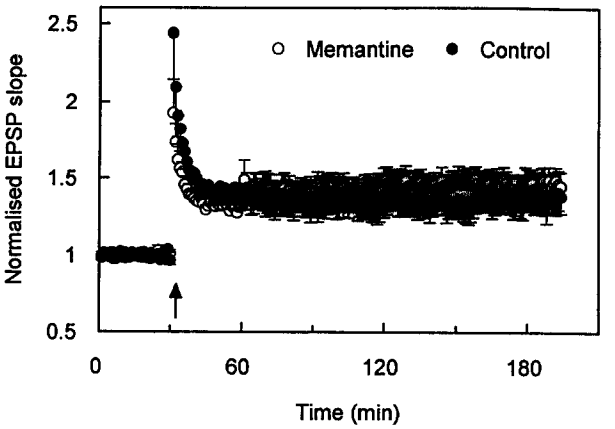

Fig. 5. Chronic infusion of memantine did not influence the induction of LTP. Each point is the mean ± S.E.M. of the field EPSP slope, for control ($n = 6$) and memantine ($n = 6$). High frequency stimulation (100 Hz, 1 s) was given at the time indicated by the arrow.

in slices from both groups. There was no statistically significant difference in the magnitude of long-term potentiation between memantine-treated animals and the control group (Fig. 5).

4. Discussion

The present data indicate that chronic infusion of quinolinic acid produces an impairment of T-maze alteration, indicating a deficit in short-term working memory. As shown by Misztal et al. (1994), this deficit is both progressive and persistent i.e. not seen after 3 days but evident after 2 weeks of quinolinic acid infusion and still present 2 weeks after the termination of the infusion. Parallel s.c. infusion of the NMDA receptor antagonists MK-801 and memantine prevented the development of the quinolinic acid-induced learning deficit. This confirms the neuroprotective activity of both agents. In the present study the neuroprotective effect of memantine was verified by attenuation of a decrease in [^{3}H]hemicholinium-3 binding (choline uptake sites) produced by quinolinic acid infusion. Previously Yamada et al. (1990) showed that i.c.v. infusion of quinolinic acid at the same dose produces a clear deficit in the cholinergic system, as evidenced by a decrease in choline acetyltransferase activity in the cortex and hippocampus. In a follow-up study Yamada et al. (1991) showed that rats also demonstrate a learning deficit in an active avoidance task after infusion of quinolinic acid. The T-maze alternation used in the present study offers a sensitive measure of performance without the significant emotional component present in negatively reinforced, repetitive trial procedures such as active avoidance.

It should be stressed that the neuroprotective doses of both MK-801 and memantine used in the present study blocked neither long-term potentiation ex vivo nor learning in the T-maze. This finding may at first appear to contradict the accepted role of NMDA receptors in plasticity processes including learning and long-term potentiation (Morris et al., 1986; Collingridge and Singer, 1990; Danysz et al., 1995). However, there are many examples that otherwise behaviourally effective doses of NMDA receptor antagonists fail to affect learning under some conditions (see Danysz et al., 1995 for review). In fact, positive effects of NMDA receptor antagonists on learning have also been demonstrated when the performance of control animals is poor because of low reinforcement levels (Mondadori et al., 1989) or due to entorhinal cortex lesions (Zajaczkowski et al., 1996). The positive effects of NMDA receptor antagonists always show bell-shaped dose-response curves and certainly, at sufficiently high doses, learning disruption can be seen (Mondadori et al., 1989; Danysz et al., 1995).

Specifically, memantine at a single dose of 20 mg/kg prevents ischaemia-induced damage, and a peak concentration expected to appear in the serum after this dose (6 μM, Danysz et al., 1994a) does not inhibit long-term potentiation in vitro (Stieg et al., 1993). Wenk et al. (1995) found that MK-801 and memantine block the decrease in choline acetyltransferase activity induced by direct injection of NMDA into the nucleus basalis magnocellularis, with respective ED_{50}'s of 0.07 and 2.7 mg/kg. This protective activity was also expressed in functional terms such as attenuation of a T-maze alternation deficit (Wenk et al., 1994). These doses of MK-801 and memantine are far below those required (0.2 and 20 mg/kg respectively) to impair learning (Misztal and Danysz, 1995).

Hence, the question arises why some NMDA receptor antagonists at doses that are neuroprotective do not affect learning or long-term potentiation. This could be due to the voltage dependence of NMDA channel blockers, meaning that the block is removed upon depolarization (Honey et al., 1985; Parsons et al., 1993). It is possible that during long-term potentiation the degree of depolarization is higher, although of shorter duration than during excitotoxicity e.g. in ischaemia or induced by direct application of an agonist (Globus et al., 1988; Clements et al., 1992).

The present study failed to provide evidence supporting the superiority of 'low affinity' NMDA channel blockers (such as memantine) over those having high affinity (such as MK-801), as suggested by some authors (Chen et al., 1992; Rogawski, 1993; Parsons et al., 1993). However, Zajaczkowski et al. (1996) observed a clear disruption of reference memory in more difficult tasks such as the radial maze after treatment of naive rats with the same dose of MK-801, but not memantine. The T-maze test procedure used in the present study measures short-term working memory, which is less sensitive to NMDA antagonists than is reference memory (Danysz et al., 1995).

The dose of memantine used (20 mg/kg per day) was selected on the basis of preliminary studies since it leads to pseudo steady-state levels in serum of 1.2 μM i.e. close to a therapeutic range in demented patients treated with memantine (Kornhuber et al., 1994). This level was reached as soon as 12 h after the start of the infusion. At this concentration the effective and selective blockade of NMDA receptors might be expected (Parsons et al., 1993; Kornhuber et al., 1994). This might indicate that neuroprotective activity of memantine can be expected in demented patients at the clinically used doses, if the excitotoxic hypothesis of dementia is accepted (Greenamyre et al., 1988).

MK-801 was used at a dose 70 times lower than memantine, based on differences in in vitro and in vivo potency (Bisaga et al., 1993; Parsons et al., 1993; Danysz et al., 1994a; Kornhuber et al., 1994) and considering similar half-lives after acute i.p. injection (Vezzani et al., 1989; Wenk et al., 1994). The only clinical study with MK-801 showed moderate therapeutic efficacy in epilepsy (Leppik et al., 1988), but in long-term treated patients serum concentrations were as high as 1.7 μM. This is far above the concentration needed to block NMDA receptors in preclinical models (Wong et al., 1988; Willis et al., 1991; Parsons et al., 1993). Thus, it is not clear what doses of MK-801 in animals might be considered 'therapeutically relevant'.

In the present study and in a previous report (Danysz et al., 1994a) the memantine concentration in the brain homogenates was much higher than in serum i.e. 40 μmol/kg tissue. This might be due to the accumulation of memantine in lysosomes (Honegger et al., 1993), but it is not necessarily indicative of similar effective concentrations in the synaptic cleft. In fact, when the interstitial concentration is assessed in the brain by microdialysis, free plasma and cerebrospinal fluid (CSF) concentrations are very close to each other (Spanagel et al., 1994). Such an accumulation in brain tissue homogenates is also evident for MK-801, but as for memantine, CSF and plasma concentrations are similar (Vezzani et al., 1989; Willis et al., 1991).

In conclusion, the present data indicate that the uncompetitive NMDA receptor antagonists MK-801 and memantine show neuroprotective activity functionally expressed in a learning task in rats receiving chronic i.c.v. infusion of quinolinic acid. The neuroprotective doses of both antagonists failed to affect learning or long-term potentiation ex vivo in naive animals indicating that, under some circumstances, it is possible to obtain neuroprotective activity with NMDA receptor antagonists without amnesia. On the basis of previous studies from our laboratory it seems that the therapeu-

tic index of memantine is indeed better than that of MK-801 (Wenk et al., 1995; Zajaczkowski et al., 1996) but the present study did not address this question directly.

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