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Adaptations of NMDA and dopamine D_2, but not of muscarinic receptors following 14 days administration of uncompetitive NMDA receptor antagonists

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Summary. Behavioral changes have previously been reported following administrations of uncompetitive NMDA receptor antagonists memantine, amantadine and MK-801 for 14 days, at the doses that produce plasma levels comparable to those seen in patients (20, 100 and 0.31 mg/kg/day respectively). Using the same doses, the effect on receptor binding (autoradiography) was studied in rats. [^{3}H]MK-801 binding was increased in the dentate gyrus and CA3 region of the hippocampus (35.2 and 24.3% respectively) following 3 days S.C. infusion of memantine by ALZET minipumps. One daily injection of memantine for 14 days, increased [^{3}H]MK-801 binding in the frontal cortex by 40.3%. The same treatment with amantadine did increase [^{3}H]raclopride binding to dopamine D_2 receptors by 13.5%. None of these treatments changed the expression of muscarinic receptors. It is concluded that subchronic blockade of the NMDA receptor by uncompetitive antagonists at moderate (therapeutically-relevant) doses induced only minor changes in NMDA and dopamine D_2 receptor expression.

Keywords: Amantadine, dopamine D_2 receptor, memantine, (+)MK-801, muscarinic receptor, NMDA receptor, receptor autoradiography.

Introduction

Uncompetitive antagonists of the N-methyl-D-aspartate (NMDA) receptor bind to the so-called PCP (phencyclidine) site inside the cationic channel of this receptor (Anis et al., 1983; Honey et al., 1985). The aminoadamantanes memantine and amantadine block this channel with a relatively moderate affinity in a use and voltage-dependent fashion (Kornhuber et al., 1991; Parsons et al., 1993). Thus, they show a favorable side-effect profile and are used clinically in the treatment of Parkinson's disease and dementia (Danysz

et al., 1997; Kornhuber et al., 1994). (+)MK-801 (dizocilpine) also blocks the NMDA receptor in an uncompetitive manner but possesses a higher affinity for this channel (Parsons et al., 1993). This taken together with its relatively slow blocking and unblocking kinetics precludes (+)MK-801's clinical use because of its pronounced side-effects (Parsons et al., 1993).

Uncompetitive NMDA receptor antagonists show a wide variety of possible therapeutical applications, which include; epilepsy, chronic pain, anxiety, morphine tolerance, drug-dependence, and neuroprotection in chronic neurodegeneration as seen in Parkinson's and Alzheimer's disease (Danysz et al., 1995; Kalivas, 1995; Löscher, 1993; Stephens, 1995; Trujillo and Akil, 1991). As stated previously, memantine and amantadine are already in clinical use for the treatment of Parkinson's disease and dementia respectively (Danysz et al., 1997; Ditzler, 1991; Kornhuber et al., 1994; Schwab et al., 1969). These indications involve prolonged administration of these drugs. For a wide range of compounds it has been shown that prolonged administration can result in adaptive changes in neurotransmitter receptor systems affected directly by the drug or down the stream. This is of special interest in case of the glutamatergic system which interacts with a number of other neurotransmitters, e.g. dopamine, GABA, acetylcholine, and noradrenaline (Schmidt, 1995), to affect numerous physiological, e.g. movement, learning, and memory formation, as well as pathological processes (Danysz et al., 1995).

Moreover, glutamatergic neurotransmission, and especially that mediated by NMDA receptors, has been implicated to play a major role in the development of synaptic plasticity during neuronal development, in learning, i.e. long-term potentiation (LTP), as well as in kindling (Collingridge and Singer, 1990; Löscher, 1993). Similarly, changes of NMDA receptor function have been related to tolerance, sensitization, and physical dependence following chronic drug administration (Kalivas, 1995; Stephens, 1995; Trujillo and Akil, 1995; Wolf, 1998). For this reason it was of interest to determine whether prolonged administration of the uncompetitive NMDA receptor antagonists memantine, amantadine and (+)MK-801, would affect NMDA receptor expression. From reports in the literature it is known that prolonged administration of the high-affinity uncompetitive NMDA receptor antagonists (+)MK-801 and ketamine is able to influence the expression of both NMDA, muscarinic as well as dopamine D_2 receptors (Beart and Lodge, 1990; Micheletti et al., 1992; Morita et al., 1995).

Previously, the effects of sub-chronic treatment with amantadine, memantine and (+)MK-801 on the typical behavioral profile of these compounds was determined. It was shown that tolerance developed to the ataxic effect and the acute learning impairment caused by memantine and (+)MK-801 (Hesselink et al., 1999). Moreover, sensitization was observed to the hyperlocomotor effect of memantine, both in intact animals in the open-field and in rotations in 6-OHDA lesioned animals. The anticataleptic effect of amantadine remained unchanged (Hesselink et al., 1999). In the experiments described here the same dose-regimens were used in order to verify whether changes in receptor expression underlie the described changes in behavior.

The objective of this study was to establish whether adaptive changes in the expression of NMDA, dopamine D_2 and muscarinic receptors occur following sub-chronic treatment with memantine, amantadine and (+)MK-801 at therapeutically relevant doses in rats. If so, do these adaptive changes develop the same way when the drugs are infused continuously or injected repetitively? This last issue is of major concern in case of the aminoadamantanes, since their elimination half-life is considerably longer in humans (up to 100 hr.) than in rodents (2–6 hr.). In the clinical situation, depending on dosing schedule, only minor fluctuations in the steady-state plasma levels of the aminoadamantanes are observed. The only way to mimic this in animals is by continuous infusion of the drug using ALZET osmotic minipumps. However, for the sake of comparison to the literature, the drugs were also repeatedly injected in this study. The importance of using these different modes of administration arises from the fact that, for a number of compounds, it is has been reported that either sensitization or tolerance can develop depending on the dose or the dose-regimen used, i.e. repetitive versus continuous administration (Kalivas, 1995; Stephens, 1995).

Materials and methods

Subjects

Adult male Sprague Dawley rats (body weight 235–275 g.), obtained from Charles River (Germany), had free access to food and water. During their treatment period, the food was restricted to 15 g/day the first week and 20 g/day the second week. The animals were kept under standard laboratory conditions: 12/12 hr. dark/light cycle, 20°C.

Treatment

Subcutaneous infusions: ALZET osmotic minipumps (ALZA corporation, Palo Alto, USA, model 2ML2) were filled with a memantine amantadine or MK-801 solution in water delivering a dose of 20, 100 or 0.31 mg/kg/day. Pump implantation was performed under Hypnorm anesthesia (0.25 ml/kg, Janssen Pharmaceuticals). A 1.5 cm. long cut was made in skin in the neck of the animal, the ALZET pump was introduced and the skin was closed using wound clamps. The experiments consisted of three treatment groups for each compound tested (n = 6–8). In the first group (14 days infusion) the pumps were implanted on day −14, a sham surgery was done on day −4, the pumps were removed on day −1 and the experiment was performed on day 0. In the second group (3 days infusion) a sham surgery was done on day −14, the pumps were implanted on day −4 and removed on day −1. The last group (sham) underwent 3 sham surgeries.

Subcutaneous injections: The animals (n = 6–8 per group) received daily subcutaneous injections of memantine, amantadine, MK-801 or saline in the same dose as the animals who received their treatment by sc. infusion (20, 100 or 0.31 mg/kg/day respectively).

Tissue preparation

The animals were decapitated, their brains were removed rapidly and frozen in 2-methylbutane cooled on dry ice. Serial 16 μm thick sections were cut on a Reichert-Jung cryostat (2800 Frigocut) and thaw-mounted on gelatin-coated slides. The sections were cut horizontally with a dorso-ventral distance from the plane passing through bregma and lambda on the top of the skull, ranging from −5.10 to −7.60 mm. Triplicate slides for assay of total and duplicate slides for the assay of non-specific binding for each ligand were cut. The slides were stored at −80°C until analysis.

Receptor autoradiography

Quantitative autoradiography for muscarinic, dopamine D_2 and NMDA receptors was performed with [^{3}H]QNB (l-quininuclidinyl[phenyl-4-^{3}H]benzilate) (Murase et al., 1991), [^{3}H]raclopride (Lidow et al., 1991), and [^{3}H]MK-801 (Sakurai et al., 1991) respectively using conventional techniques.

All assays were performed in an identical manner with specifications given in Table 1. Slides were warmed to room temperature and allowed to dry. Subsequently they were immersed in washing buffer and dried under a cold stream of air. The sections were placed in incubation buffer (70 ml) containing a single concentration of radioactive ligand. For determination of non-specific binding the slides were incubated in the ligand solution containing the appropriate blocker. Incubation was terminated by removal of the slides from the ligand medium and rapid washing as indicated in Table 1. The slides were then allowed to dry and subsequently the dried sections and tritium standards (Microscales, Amersham) were opposed to tritium sensitive films (^{3}H-hyperfilms, Amersham) in light-tight cassettes. After 2–8 weeks, the films were developed using Kodak developer D-19.

Ligand binding was quantified with a Macintosh computer assisted densitometry analyzing system (NIH Image, Version 1.51). To quantify ligand binding density, the optical density of co-exposed standards was determined and fitting standard values with a third degree polynomial regression equation generated a standard curve. Use of the standards allowed conversion of areal optical density into μCi/mg tissue values. All brain regions were read with a variable size cursor to allow sampling of the entire area of the brain structure of interest.

Table 1. Specifications of the autoradiographic procedures for [^{3}H]QNB (l-quininuclidinyl[phenyl-4-^{3}H]benzilate) (Murase et al., 1991), [^{3}H]raclopride (Lidow et al., 1991), and [^{3}H]MK-801 (Sakurai et al., 1991) binding to brain tissue slices

Receptor	Pre-washing	Ligand	Incubation conditions	Non-specific binding	Post-washing
Muscarinic	2 × 15 min. at 22°C in buffer	[^{3}H]QNB 1.5 nM	120 min. at RT, 50 mM Tris-HCl (pH = 7.4)	1 μM atropine	3 × 10 sec. in ice-cold buffer followed by water
Dopamine D_2	20 min. at 22°C in buffer with 150 mM NaCl	[^{3}H]raclopride 1 nM	45 min at RT in buffer with 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid, 50 mM Tris-HCl (pH = 7.4)	2 μM butaclamol	6× in ice-cold buffer followed by water
NMDA channel site	2 × 15 min. at 4°C in buffer	[^{3}H]MK-801 5 nM	120 min at RT in buffer containing 10 μM glycine and 10 μM L-glutamate, 5 mM Tris- HCl (pH = 7.4)	10 μM (+)MK-801	2 dips in 4°C buffer, 4°C water followed by 1.25% glutaraldehyde in acetone

Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was performed using ANOVA followed by the Benferroni's test when applicable (SigmaStat, Jandel, USA).

Chemicals

The following substances were used: 1-amino-3,5-dimethyladamantane HCl (memantine, Merz + Co, Germany), 1-aminoadamantane HCl (amantadine, Aldrich, Germany), butaclamol (RBI, USA), atropine (RBI, USA), [^{3}H]QNB (l-quininuclidinyl[phenyl-4-^{3}H]benzilate) (40 Ci/mmol, Dupont, Germany), [^{3}H]raclopride (81 Ci/mmol, NEN, Germany), and [^{3}H]MK-801 (23.9 Ci/mmol, Dupont, Germany)

Results

[^{3}H]MK-801

The binding of [^{3}H]MK-801 to the channel site of the NMDA receptor was determined in the entorhinal cortex, dentate gyrus (DG), the CA1 and CA3 regions of the hippocampus, caudate putamen, nucleus accumbens, and the frontal cortex (Fig. 1A–G). Binding densities were highest in the hippocampal areas (DG, CA1 and CA3) compared to all other brain structures measured. When determining the effect of continuous NMDA receptor blockade with either memantine, amantadine or (+)MK-801, the only effect that was observed was an increase of [^{3}H]MK-801 binding following 3 days of amantadine infusion (100 mg/kg/day). This increase reached significance in the dentate gyrus (35.2%, Fig. 1B) and CA3 region of the hippocampus (24.3%, Fig. 1D), while in all other brain structures measured this change was only detected as a trend towards an increase (Fig. 1A, C, E–G). Following 14 days amantadine infusion this increased binding density was no longer observed. Neither 3 nor 14 days infusion of memantine (20 mg/kg/day) or (+)MK-801 (0.31 mg/kg/day) had an effect on [^{3}H]MK-801 binding.

Following 14 days of repeated administration, memantine (20 mg/kg once daily, sc.) increased [^{3}H]MK-801 binding in the frontal cortex by 40.3% (Fig. 1G). This effect of memantine was specific for the frontal cortex since it was not observed in any of the other brain structures measured (Fig. 1A–F). Repeated administration of either amantadine or (+)MK-801 (100 and 0.31 mg/kg once daily sc. for 14 days respectively) was unable to affect [^{3}H]MK-801 binding in any of the brain structures measured (Fig. 1A–G).

[^{3}H]raclopride

[^{3}H]raclopride binding to dopamine D_2 receptors in the caudate putamen and nucleus accumbens was comparable (Fig. 2A + B). Infusion of memantine, amantadine or (+)MK-801 (20, 100, and 0.31 mg/kg/day respectively) for either 3 or 14 days did not affect [^{3}H]raclopride binding to brain slices (Fig. 2). Repeated injections of amantadine (100 mg/kg once daily, sc.) however, caused a minor (13.5%) but significant increase in [^{3}H]raclopride binding in

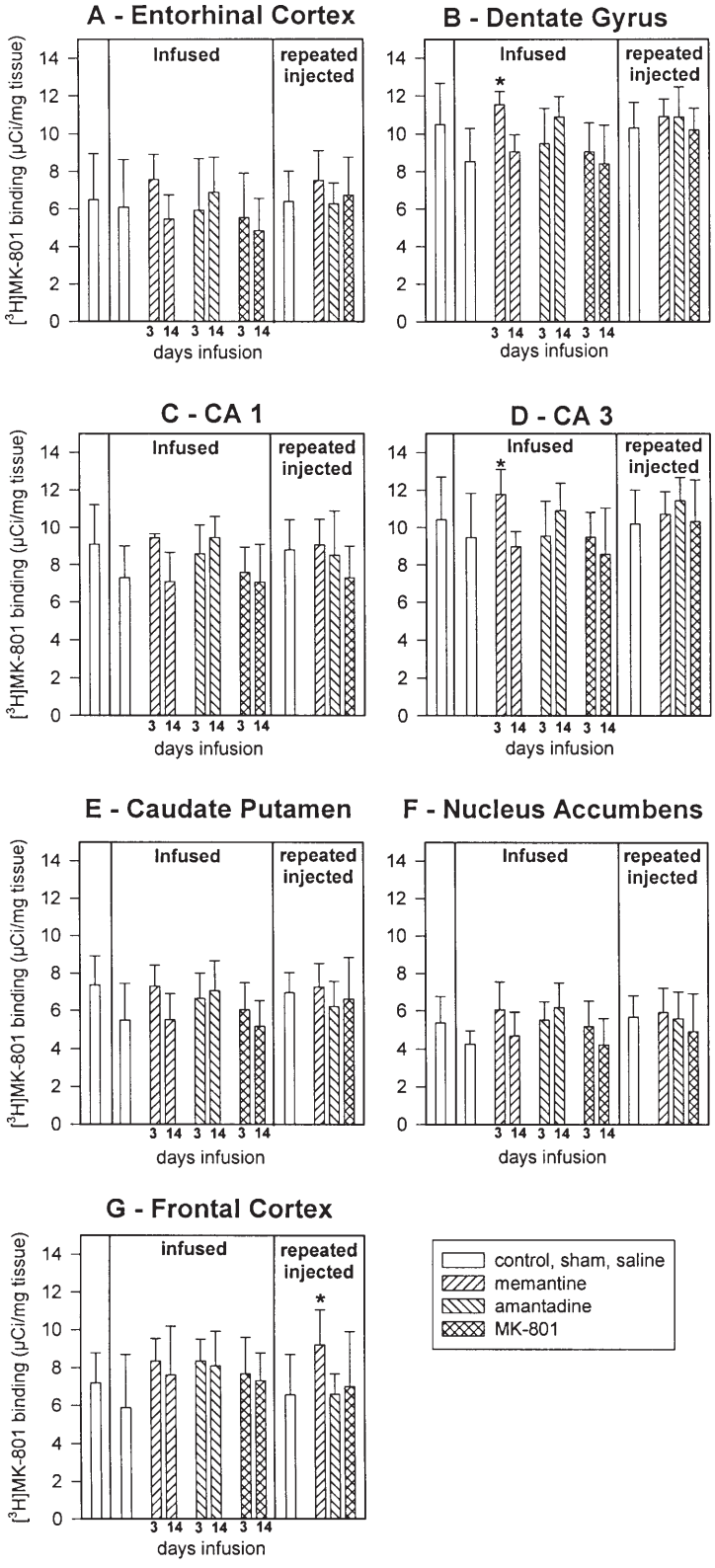

Fig. 1. Characterization of [^{3}H]MK-801 autoradiography in the entorhinal cortex (**A**), dentate gyrus (**B**), CA1 (**C**) and CA3 (**D**) region of the hippocampus, caudate putamen (**E**) nucleus accumbens (**F**) and frontal cortex (**G**) (µCi/mg tissue) in animals treated with memantine, amantadine or MK-801 (20, 100 or 0.31 mg/kg) for 3 or 14 days by continuous sc. infusion using ALZET osmotic minipumps or repeated s.c. injections for 14 days. Data are mean ± SEM. N = 6–8. *−$p < 0.05$ vs. respective control (sham or saline)

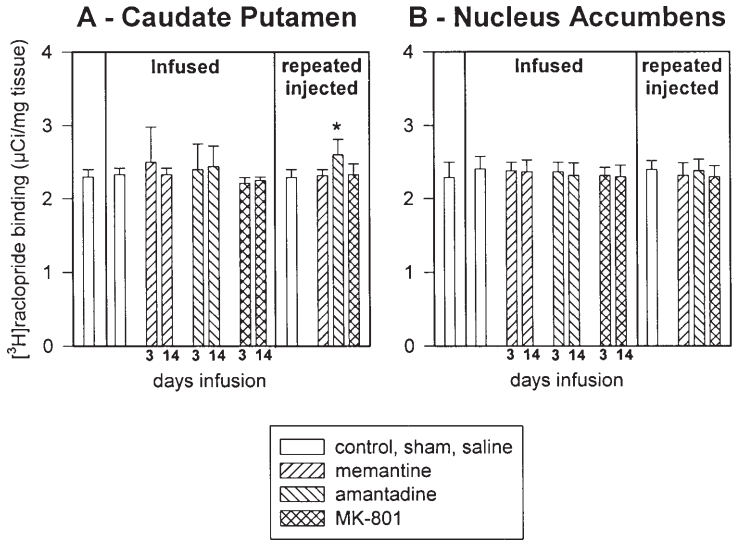

Fig. 2. [^{3}H]raclopride binding in the caudate putamen (**A**) and nucleus accumbens (**B**) (μCi/mg tissue) to brain tissue slices (16 μm) in animals treated with memantine, amantadine or MK-801 (20, 100 or 0.31 mg/kg) for 3 or 14 days by continuous sc. infusion or repeated s.c. injections for 14 days. Data are mean ± SEM. N = 6–8

the caudate putamen (Fig. 2A). Repeated injections of either memantine or (+)MK-801 (20 mg/kg once daily, sc.) failed to increase [^{3}H]raclopride to dopamine D_2 receptors.

[^{3}H]QNB

[^{3}H]QNB binding to muscarinic receptors was highest in the dentate gyrus and the CA3 region of the hippocampus, when compared to binding densities in the frontal and entorhinal cortices and the CA1 region of the hippocampus (Fig. 3A–E). The binding of [^{3}H]QNB to muscarinic receptors was not affected by sub-chronic administration of any of the uncompetitive NMDA receptor antagonists, memantine, amantadine or (+)MK-801 (20, 100 and 0.31 mg/kg/day respectively) given either by continuous infusion for 3 or 14 days or by repeated injection for 14 consecutive days.

Discussion

In the experiments described in the present report, the doses of memantine, amantadine and MK-801 used were aimed to be of clinical relevance. In case of memantine, within 2 days after implantation of the ALZET osmotic minipumps, steady-state plasma levels develop which are comparable to the plasma levels seen in patients, c.a. 1 μM (Danysz et al., 1997; Kornhuber and Quack, 1995; Kornhuber et al., 1994). Moreover, this dose has been reported to produce steady-state brain extracellular fluid concentrations within range of memantine's affinity for the NMDA receptor (Hesselink et al., 1997). For amantadine the dose was chosen based on plasma concentrations in patients

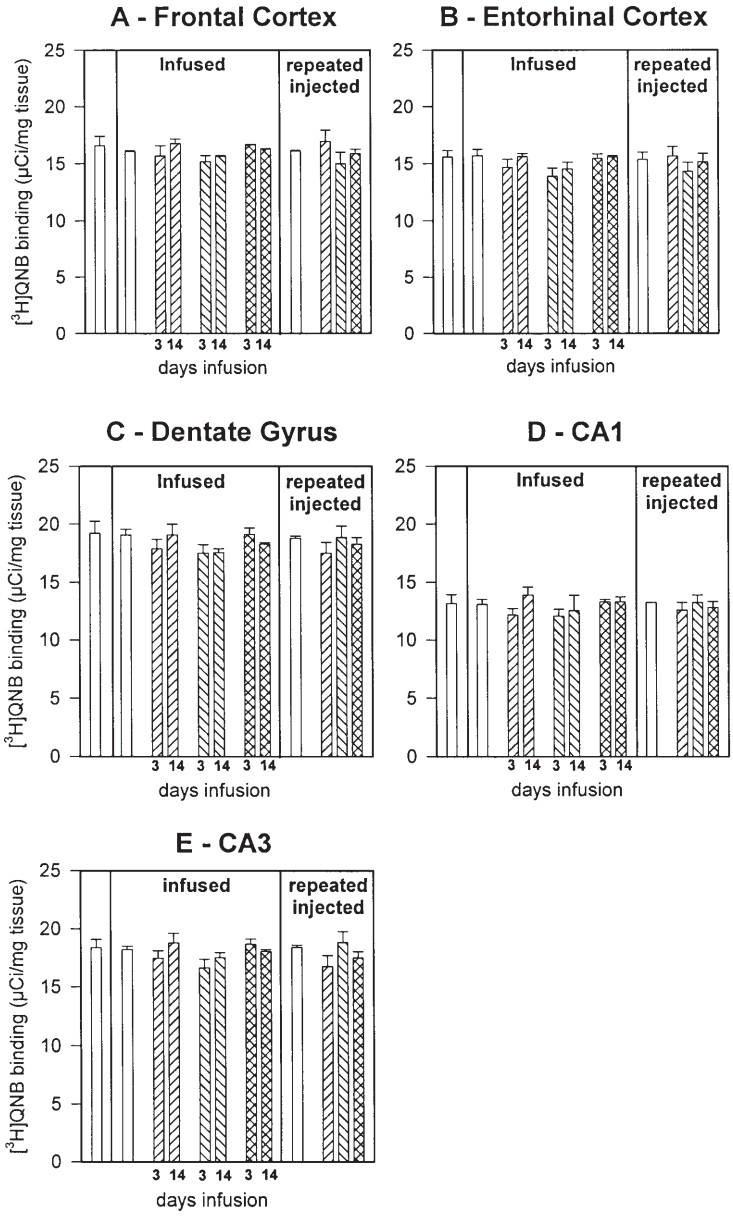

Fig. 3. [^{3}H]QNB binding in the frontal cortex (**A**), entorhinal cortex (**B**), dentate gyrus (**C**), CA1 (**D**) and CA3 (**E**) region of the hippocampus (µCi/mg tissue) to brain tissue slices of animals treated with memantine, amantadine or MK-801 (20, 100 or 0.31 mg/kg) for 3 or 14 days by continuous sc. infusion using ALZET osmotic minipumps or repeated s.c. injections for 14 days. Data are mean ± SEM. N = 6–8

which were 5–10 times higher than memantine concentrations (Danysz et al., 1997; Kornhuber and Quack, 1995). However, the brain ECF concentration following this dose is lower than amantadine's affinity for the NMDA receptor (Hesselink et al., 1997). Thus while in case of memantine we can consider NMDA antagonism as a major (if not only) action at therapeutically relevant

doses (and in the present study), this does not seem to hold true for amantadine. In fact other targets that come into question are: nicotinic receptors, sigma-1 receptors, aromatic amino acids decarboxylase, brain 60 kDa calmodulin-dependent cyclic nucleotide phosphodiesterase, super oxide dismutase, and GFAP (Danysz et al., 1997; Kakkar et al., 1997). Both aminoadamantanes have a long plasma half-life in patients (up to 100 hr. for memantine) and show minor fluctuations in steady-state plasma levels. Since the plasma elimination half-life of these compounds in rodents is considerably shorter (2–3 hr), osmotic minipumps were chosen for their steady state delivery. In case of (+)MK-801, the dose was selected on the basis of an initial clinical report on the use of (+)MK-801 as an anti-epileptic drug and on pharmacokinetic studies in animals (Leppik et al., 1988; Schwartz and Wasterlain, 1993; Vezzani et al., 1989). In rats an i.v. bolus (0.12 mg/kg) followed by an i.v. infusion of 0.108 mg/kg/hr, produced a plasma concentration of 50 nM (Willis et al., 1991). In patients, a plasma concentration of 1.44 nM resulted in >50% reduction in seizures (Leppik et al., 1988), indicating that much lower doses are therapeutically relevant. In the experiments presented here (+)MK-801 was infused s.c. instead of i.v. Since the bioavailability of (+)MK-801 is not known following s.c. infusion, a dose of 0.31 mg/kg/day was used. This dose was previously shown to be neuroprotective against both acute administration of NMDA into the nucleus basalis magnocellularis and chronic i.c.v. infusion of quinolinic acid without any overt behavioral effects as ataxia or locomotor activation (Misztal et al., 1996; Wenk et al., 1997). For the sake of comparison to existing literature, the tested agents were also administered as repeated s.c. injections. By comparing these dose-regimens it might be possible to ascertain whether continuous, non-fluctuating steady state levels or high peak concentrations are more important in inducing changes in receptor expression.

Sub-chronic treatment with uncompetitive NMDA receptor antagonists only moderately increased dopamine D_2 and NMDA receptor expression (amantadine and memantine respectively), while muscarinic receptor expression remained unaltered. NMDA receptor expression showed a minor increase in the dentate gyrus and the CA3 region of the hippocampus following 3 days of continuous infusion of memantine. This increase was not observed anymore following 14 days of infusion. This would indicate that this alteration is only transient. The increased expression of the glutamate-site of the NMDA receptor has been described following 7 days treatment with (+)MK-801 (0.5 mg/kg twice daily) (Beart and Lodge, 1990; Manallack et al., 1989). However, Kurumaji and coworkers (1991) did not observe any change in NMDA receptor expression following administration of the same dose for 14 days. This would lead to the conclusion that any initial increase in expression in the glutamate binding-site of the NMDA receptor could indeed be transient. Moreover, in case of the NMDA receptor a distinction has to be made between the different binding sites on this receptor. The earlier mentioned studies by Beart and Lodge (1990) and Manallack and colleagues (1989), did indeed find an increased expression of the glutamate binding-site but not the channel-site of this receptor. This can be explained by different regulation of

the expression of NMDA receptor subunits. Moreover, in the experiments presented in the present paper, simple autoradiography procedure was used to determine NMDA receptor expression (one ligand concentration, one concentration of the displacer). Thus in the present study any changes of specific binding could be due to alterations in affinity as well as Bmax. Binding-site density can be also influenced by alterations in subunit composition. In case of the NMDA receptor it is known that the different binding-sites are located at different subunits. Thus, it can not be excluded that binding to other recognition sites would be affected differently.

In the present study, [^{3}H]MK-801 binding to NMDA receptors in the prefrontal cortex was moderately increased following 14 days of memantine injections. Previously, it had been shown that sensitization developed to the hyperlocomotor activity induced by memantine (but not MK-801) in intact animals following 14 days repeated injections with the same dose (Hesselink et al., 1999). It can be argued that this increase in NMDA receptors in the prefrontal cortex would explain this sensitization since it was only seen following 14 days injections and not following infusion of the same dose (Hesselink et al., 1999). Previously Bresink and colleagues (1995) reported a similar effect – increase in [^{3}H]MK-801 binding – following long term (20 month) treatment with memantine. In this case the study was performed on cortical homogenates and revealed an increase in Bmax without change in Kd.

Regarding the dopaminergic system, it has been previously demonstrated that prolonged (50 days) exposure of rats to either ketamine or (+)MK-801 increases the expression of dopamine D_2 receptors on medium sized spiny neurons in the striatum by a recruitment phenomenon. This means that a greater number of neurons express dopamine D_2 receptors than before treatment started (Lannes et al., 1995; Micheletti et al., 1992). However, in our experiments neither memantine nor (+)MK-801 affected dopamine D_2 receptors in the striatum. It could be argued that the duration of treatment in the present experiments was not sufficient to exert this effect. Moreover, in most experiments described in literature, higher doses (0.8 to 0.96 mg/kg/day) of (+)MK-801 were used (Qin et al., 1994; Lannes et al., 1995). Micheletti and coworkers (1992) used (+)MK-801 in a dose-range of 0.1 to 0.4 mg/kg and found only an effect at the highest dose. The (+)MK-801 dose used in this study was lower (0.31 mg/kg/day). In the studies presented in this paper, a minor (13.5%) increase in caudate putamen [^{3}H]raclopride binding to dopamine D_2-receptors was observed following 14 days of repeated injections with amantadine (100 mg/kg/day).

Following treatments used in this study, no change was observed in the expression of muscarinic cholinergic receptors, as determined by [^{3}H]QNB binding to frontal cortex, entorhinal cortex, dentate gyrus and CA1 and CA3 region of the hippocampus. Morita and colleagues (1995) have previously shown that administration of ketamine (25 mg/kg) every three days for a total of 5 days increased [^{3}H]QNB binding in the forebrain of mice. This up-regulation of muscarinic receptors was accompanied by a reduced scopolamine-induced hyperlocomotion.

It could be argued that the doses used in these experiments are relatively moderate which leads to no or only minor changes in NMDA, dopamine D2 and muscarinic receptor. However, in the case of memantine and amantadine these doses produce plasma concentrations comparable to those seen in humans (Danysz et al., 1997; Hesselink et al., 1997; Kornhuber and Quack, 1995; Kornhuber et al., 1994). In the case of memantine it has been shown that infusion of 20 mg/kg/day results in a brain ECF concentration within the range of memantine's in vitro affinity for the NMDA receptor (Hesselink et al., 1999). Moreover, behavioral changes have been observed following 14 daily injections or infusion of memantine and (+)MK-801 at the doses used in this study. As previously mentioned, sensitization developed to memantine's locomotor stimulatory effect, which could possibly be explained by the increase in NMDA receptors in the prefrontal cortex following this treatment. However, the development of tolerance to the ataxic and learning impairing properties of the uncompetitive NMDA receptor antagonists can not be explained on the basis of changes in receptor expression seen in this study. Tolerance to these effects developed for both memantine and (+)MK-801 following 14 but not 3 days administration (Hesselink et al., 1999).

In general, it can be concluded that the effect of 14 days treatment with memantine, amantadine or (+)MK-801 in therapeutically relevant doses produced only minor changes in expression of NMDA and dopamine D_2 receptors, and no effect on muscarinic receptor. In particular the changes after infusion leading to steady state levels – which is therapeutically more relevant – were negligible.

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