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Are neuronal nicotinic receptors a target for antiepileptic drug development? Studies in different seizure models in mice and rats

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Abstract

Altered function of neuronal nicotinic acetylcholine receptors in the brain has recently been associated with an idiopathic form of partial epilepsy, suggesting that functional alterations of these receptors can be involved in the processes leading to epileptic seizures. Thus, nicotinic acetylcholine receptors may form a novel target for antiepileptic drug development. In the present study, various nicotinic acetylcholine receptor antagonists, including novel amino-alkyl-cyclohexane derivatives, were evaluated in two animal models, namely the maximal electroshock seizure test in mice and amygdala-kindling in rats. For comparison with these standard models of generalized and partial seizures, the effects against nicotine-induced seizures were examined. Because some of the agents tested showed an overlap between channel blocking at nicotinic acetylcholine receptors and NMDA receptors, the potency at these receptors was assessed by using patch clamp in a hippocampal cell preparation. Preferential nicotinic acetylcholine receptor antagonists were potent anticonvulsants in the maximal electroshock seizure test and against nicotine-induced seizures. The anticonvulsant potency in the maximal electroshock seizure test was decreased by administration of a subconvulsant dose of nicotine. Such a potency shift was also seen with selective NMDA receptor antagonists, which were also efficacious anticonvulsants against both maximal electroshock seizures and nicotine-induced seizures. Experiments with agents combining nicotinic acetylcholine receptor and NMDA receptor antagonistic effects suggested that both mechanisms contributed to the anticonvulsant effect of the respective agents in the maximal electroshock seizure test. This was not found in kindled rats, in which nicotinic acetylcholine receptor antagonists exerted less robust effects. In conclusion, it may be suggested that nicotinic acetylcholine receptor antagonism might be a valuable therapeutic approach to treat generalized epileptic seizures but rather not complex partial seizures.

Keywords: Kindling; Seizure, nicotine-induced; Nicotinic receptor; NMDA receptor; Antiepileptic drug; Epilepsy

1. Introduction

Neuronal nicotinic acetylcholine receptors represent a large family of ligand-gated ion channels composed of pentameric combinations of α and β subunits with specific structural, functional and pharmacological properties (Jones et al., 1999; Dani, 2001). Although these receptors are found in most parts of the brain, the precise physiological functions of brain nicotinic acetylcholine receptors are not well defined to date. Presynaptic and preterminal nicotinic acetylcholine receptors enhance neurotransmitter release, while postsynaptic receptors mediate fast acetylcholine-mediated excitatory synaptic transmission, at least in the hippocampus (Jones et al., 1999; Dani, 2001). Behavioral studies indicate that brain nicotinic acetylcholine receptors participate in complex functions such as attention, memory, and cognition, and clinical data suggest their involvement in the pathogenesis of a number of neuropsychiatric disorders, including drug addiction, Alzheimer's disease, schizophrenia and epilepsy (Jones et al., 1999; Court et al., 2000; Dani, 2001).

Epilepsy was the first disease for which a link with a mutated nicotinic acetylcholine receptor was found (for review, see Steinlein, 1998; Bertrand, 1999; Berkovic and Scheffer, 2001). In an idiopathic type of epilepsy, autosomal

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dominant nocturnal frontal lobe epilepsy, that displays mendelian inheritance, five distinct mutations were found in the two genes coding for the α4β2 subtype of nicotinic acetylcholine receptors, indicating that alteration of these receptors may be at the origin of the neuronal network dysfunction that causes the epileptic seizures. Recent analyses of functional properties of four nicotinic acetylcholine receptor mutants associated with autosomal dominant nocturnal frontal lobe epilepsy indicate that the sole common trait between these mutants is an increased sensitivity to acetylcholine, resulting in a gain of function of these mutant receptors (Moulard et al., 2001; Bertrand et al., 2002). In addition, there have been reports that other familial forms of epilepsy might be linked to the α3, α5, α7, and β4 subunits of nicotinic acetylcholine receptors (Jones et al., 1999). However, although these findings point to a role of nicotinic acetylcholine receptors in the modulation of brain excitability, little is known about the functional role of these receptors in the generation of seizures.

A role of the cholinergic system in convulsive disorders has long been suggested, but most studies in this respect have concentrated on muscarinic rather than nicotinic receptors (Bianchi and Beani, 1998; Turski et al., 1989; Jarrot, 1999). High systemic doses of nicotine induce clonic–tonic seizures which originate in the hippocampus, are mediated by neuronal nicotinic receptors, and are blocked by nicotinic antagonists such as mecamylamine (Stumpf and Gogolak, 1967; Dixit et al., 1971; Caulfield and Higgins, 1983; Damaj et al., 1999). Recently, release of glutamate via stimulation of presynaptic nicotinic acetylcholine receptors has been implicated in the convulsant action of nicotine, and MK-801 (dizocilpine), a potent antagonist at the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors, was found to suppress the development of nicotine-induced seizures in mice (Damaj et al., 1999).

The aim of the present study was to evaluate the effects of antagonists of nicotinic acetylcholine receptors in two standard models of epileptic seizures, the maximal electroshock seizure test in mice and the kindling model in rats. While the maximal electroshock seizure test is a model of generalized tonic–clonic seizures, kindling is a widely used model for partial seizures (Löscher, 1999). For comparison, the effect of the antagonists on nicotine-induced seizures was studied. Nicotine was also used to examine whether the anticonvulsant effect of the antinicotinic drugs in the maximal electroshock seizure test can be counteracted by addition of nicotine. In addition to well-known nicotinic antagonists such as mecamylamine, which blocks most subtypes of neuronal nicotinic receptors, and the α7-subunit selective antagonist methyllycaconitine, a novel group of amino-alkyl-cyclohexanes with antagonistic properties at nicotinic acetylcholine receptors was included in the experiments (Fig. 1). Because several compounds of this chemical class also block NMDA receptors, which is an effective means of blocking seizures (Löscher and Rogawski, 2002), all compounds were tested for their potency on nicotinic

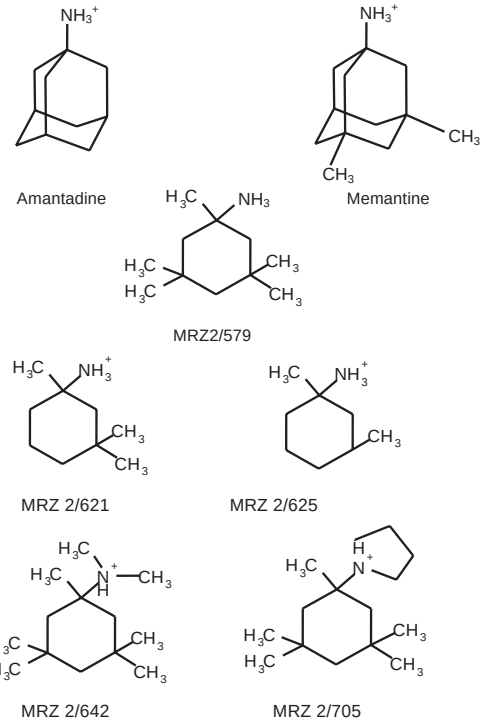

Fig. 1. Chemical structures of agents (MRZ 2/579, 2/621, 2/625, 2/642, and 2/705) from the novel series of amino-alkyl-cyclohexanes tested in this study. For comparison, structures of amantadine and memantine (1-amino-3,5-dimethyladamantane) are shown.

acetylcholine receptors and NMDA receptors in patch clamp experiments, using hippocampal neurons. Furthermore, the NMDA receptor antagonists MK-801, memantine, and amantadine were included in the study, because all three compounds have recently been reported to act as open-channel blockers at the α4β2 type of nicotinic acetylcholine receptors (Buisson and Bertrand, 1998). In addition, memantine and amantadine bear structural similarities to the novel group of amino-alkyl-cyclohexanes evaluated in the present study (Fig. 1).

2. Materials and methods

2.1. Electrophysiology

Hippocampi were obtained from rat embryos (E20–E21) and were then transferred to Ca^{2+} and Mg^{2+} free Hank's buffered salt solution (Gibco, Karlsruhe, Germany) on ice. Cells were mechanically dissociated in 0.05% DNAase/0.3% ovomucoid (Sigma, St. Louis, MO) following an 8 min pre-incubation with 0.66% trypsin/0.1% DNAase (Sigma). The dissociated cells were then centrifuged at 18 G for 10 min, re-suspended in minimum essential medium (Gibco) and plated at a density of 150,000 cells cm^{-2} onto poly-DL-ornithine (Sigma)/laminin (Gibco)-precoated plastic Petri dishes (Falcon). The cells were nourished with $NaHCO_3$/HEPES-buffered minimum essential medium sup-

plemented with 5% foetal calf serum and 5% horse serum (Gibco) and incubated at 37 °C with 5%CO_2 at 95% humidity. The medium was exchanged completely following inhibition of further glial mitosis with cytosine-β-D-arabinofuranoside (ARAC, 5 μM, Sigma) after about 5 days in vitro.

Patch clamp recordings were made from these neurones after 15–21 days in vitro with polished glass electrodes (2–3 MΩ) in the whole cell mode at room temperature (20–22 °C) with the aid of an EPC-7 amplifier (List, Darmstadt, Germany). Test substances were applied using a modified fast application system (SF-77B Fast Step, Warner Instruments, Hamden, CT, USA) with 100 μM opening diameter theta glass (Clark TGC 200-10) pulled with a Zeiss DMZ (Augsburg, Germany) horizontal puller. The contents of the intracellular solution were normally as follows (mM): CsCl (95), TEACl (20), EGTA (10), HEPES (10), $MgCl_2$ (1), $CaCl_2$ (0.2), glucose (10), Tris-ATP (5), Di-Tris-Phosphocreatinine (20), Creatine Phosphokinase (50 U); pH was adjusted to 7.3 with CsOH or HCl. CsCl was included in the intracellular solution to prevent activation of voltage activated K^+ channels in case of testing voltage dependence. The extracellular solutions had the following basic composition (mM): NaCl (140), KCl (3), $CaCl_2$ (0.2), glucose (10), HEPES (10), sucrose (4.5), tetrodotoxin (3×10^{-4}). Tetrodotoxin was used to stop spontaneous activity.

Only results from stable cells were accepted for inclusion in the analysis, i.e., showing at least 75% recovery of responses to agonist (acetylcholine or NMDA) following removal of the antagonist tested. Despite this, recovery from drug actions was not always 100% because of rundown in some cells ($\leq$ 10% over 10 min). When present, this was always compensated by basing the percentage antagonism at each concentration on both control and recovery and assuming a linear time course for this rundown. All antagonists were assessed at steady-state blockade with three to six concentrations on at least five cells. Equilibrium blockade was achieved within two to five agonist applications, depending on antagonist concentration.

2.2. Electrically and nicotine-induced convulsions in mice

2.2.1. Subjects

NMRI female mice (18–28 g) housed 5 per cage were used for the maximal electroshock and nicotine-induced convulsions and motor impairment tests. Female mice were used to be able to compare the results with previously performed experiments in our laboratory. All animals were kept with water and food ad libitum under a 12-h light–dark cycle (light on at 6 a.m.) and at a controlled temperature (20 ± 0.5 °C). Tested agents were dissolved in 0.9% saline and injected 30 min i.p. before the induction of convulsions if not otherwise stated. All experiments were performed between 10 a.m. and 5 p.m. according to the animal protection commission allowance #F 77-51 (Hessen, Germany).

2.2.2. Maximal electroshock seizure test

The maximal electroshock seizure test was performed in most cases together with tests for myorelaxant action (traction reflex) and motor coordination (rotarod). For the traction reflex test mice were placed with their forepaws on a horizontal rod and were required to place all four paws on the wire within 10 s. To test ataxia (motor coordination) mice were placed on accelerating rotarod and were required to remain on the rod for 1 min. Only mice not achieving the criteria in all three repetitions of each test were considered to exhibit myorelaxation or ataxia respectively. These tests were followed by electroshock (100 Hz, 0.5 s shock duration, 50 mA shock intensity, 0.9 ms impulse duration, Ugo Basile) applied through corneal electrodes. The presence of tonic convulsions was scored (tonic extension of hind paws with minimum angle to the body of 90°). In a set of experiments, an additional injection of a subconvulsant dose of nicotine (2 mg/kg, s.c.) was performed 15 min before the maximal electroshock seizure test in order to examine how this changed the anticonvulsant effect of the antinicotinic drugs.

2.2.3. Nicotine-induced seizures

Following nicotine injection (s.c.) mice were placed individually in the observation cages. Nicotine produced an almost immediate response consisting of wild running, clonic–tonic seizures and sometimes tonic seizures followed by death. Based on initial experiments, a nicotine dose of 8 mg/kg was selected as a reliably convulsive dose for the further interaction experiments. Seizures were scored as present (clonic or tonic) or absent starting immediately after nicotine injection and up to 5 min afterwards.

2.3. Kindling-induced convulsions in rats

2.3.1. Subjects

Female Wistar rats were purchased at a body weight of 200–220 g (Harlan-Winkelmann, Borchen, Germany) and were then kept under controlled environmental conditions (24–25 °C, 50–60% humidity, 12 h light/dark cycle) with free access to standard laboratory chow (Altromin 1324 standard diet) and tap water. Female rats were used because they are known to eliminate drugs less rapidly than do male rats and gain less body weight than male rats, which is an advantage for drug efficacy studies, particularly when new compounds with limited availability are tested. On the basis of experiments with daily stimulation of fully kindled Wistar rats, we have no indication that the estrous cycle affects the expression of kindled seizures (Wahnschaffe and Löscher, 1992), and drug studies gave no evidence that the estrous cycle affects anticonvulsant drug responses (Rundfeldt et al., 1990).

All experiments were performed within the same day time in the morning to minimize possible effects of circadian variation. During the period of experiments animals had a body weight between 255 and 419 g. All animal care

and handling was conducted in compliance with the German Animal Welfare Act and was approved by the responsible governmental agency in Hannover.

2.3.2. Electrode implantation

For implantation of kindling electrodes, rats were anaesthetized with chloral hydrate (360 mg/kg, i.p.), the skull surface was exposed, and a bipolar electrode was implanted into the right hemisphere aimed at the basolateral amygdala using the following stereotaxic coordinates according to the atlas of Paxinos and Watson (1998): 2.2 mm caudal, 4.8 mm lateral, 8.5 mm ventral (all respective to bregma). The electrodes consisted of two twisted Teflon-coated stainless steel wires (250 μm diameter) separated by 0.5 mm at the tip. A screw, which served as grounding electrode, was positioned over the left parietal cortex. Bipolar and ground electrodes were connected to plugs, and the electrode assembly and anchor screws were held in place with dental acrylic cement applied to the exposed skull surface. After surgery, the rats were treated with antibiotics for 1 week to prevent infection.

2.3.3. Kindling procedure and experiments in fully kindled animals

Following a post-operative recovery period of two weeks, constant current stimulations (500 μA, 1 ms, monophasic square-wave pulses, 50 Hz for 1 s) were delivered to the amygdala once daily (five times per week) until at least 10 sequential fully kindled stage-5 seizures were elicited. Seizure severity was scored according to Racine (1972): (1) immobility, eye closure, ear twitching, twitching of vibrissae, sniffing, facial clonus; (2) head nodding associated with more severe facial clonus; (3) clonus of one forelimb; (3.5) bilateral clonus without rearing; (4) bilateral clonus accompanied by rearing; (4.5) generalized clonic seizures without rearing and falling (e.g. because of direct loss of balance); (5) rearing and falling accompanied by generalized clonic seizures. In these fully kindled rats, the afterdischarge threshold was determined by administering a series of stimulations at intervals of 1 min increasing in steps of about 20% of the previously applied current. The afterdischarge threshold was defined as the lowest current intensity producing afterdischarge with a duration of at least 5 s. Determination of afterdischarge threshold was repeated two times to prove reproducibility before animals were used for drug testing.

In all experiments in fully kindled rats, the afterdischarge threshold and seizure parameters (seizure severity, seizure duration, afterdischarge duration) occurring at afterdischarge threshold were recorded. Seizure duration was the time period of limbic and/or motor seizures. Limbic seizure activity which sometimes occurred after termination of secondarily generalized seizures was not included in seizure duration. Afterdischarge duration was defined as the period of high amplitude spiking (at least 1 Hz frequency and twice the pre-stimulation amplitude) in the electroencephalogram (EEG) of the amygdala electrode, including the time of stimulation.

The substances mecamylamine, MK 801, nicotine, MRZ 2/625, MRZ 2/642, and MRZ 2/705 were dissolved in saline (pH 7.4) and administered i.p. in a volume of 3 ml/kg bodyweight 10 min (nicotine), 30 min (all MRZ agents) or 60 min (MK-801) before afterdischarge threshold determination. Data obtained after administration of these substances were compared with data obtained in the same rats after i.p. injection of vehicle (saline) 2–3 days before the drug experiment.

On each experimental day, kindled rats were allowed to adapt to the laboratory environment, then body temperature was measured and animals were put into open cages for constant observation. Following drug or vehicle administration, behavioural alterations and body temperature were determined. Adverse effects were scored during observation in open cages and in an open field. In addition rats were subjected to the rotarod test (polypropylene, foam-coated rod, 5 cm in diameter, 8 rpm). Animals were considered to have failed this test, when they fell from the rod in each of three consecutive 1-min attempts.

Side effects were scored as follows. Ataxia: 0 = absent, 1 = slight ataxia in hindlegs, 2 = more pronounced ataxia with dragging of hindlegs, 3 = further increase of ataxia and more pronounced dragging of hindlegs, 4 = marked ataxia and loss of balance during forward locomotion, 5 = very marked ataxia with frequent loss of balance and 6 = permanent loss of righting reflex; sedation: 0 = absent, 1 = slightly reduced forward locomotion, 2 = reduced locomotion with rest periods between periods of locomotion, 3 = reduced locomotion with more frequent rest periods and 4 = no forward locomotion and animal sits quietly with closed eyes; further adverse effects: 0 = absent, 1 = equivocal, 2 = present and 3 = intense.

2.4. Drugs

The following substances have been used in mice or rats: 1-amino-3,5-dimethyladamantane (memantine), 1-amino-1,3,3,5,5-pentamethyl-cyclohexane (MRZ 2/579; neramexane), 1-amino-1,3,3-timethyl-cyclohexane (MRZ 2/621), 1-amino-1(trans)-3-dimethyl-cyclohexane (MRZ 2/625), *N*,*N*-dimethyl-1-amino-1,3,3,5,5-pentamethyl-cyclohexane (MRZ 2/642), *N*-(1,3,3,5,5-pentamethylcyclohexyl)-pyrrolidine hydrochloride semihydrat (MRZ 2/705), (all HCl salts if not stated otherwise, Merz Pharmaceuticals, Frankfurt /Main, Germany); (+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate (MK-801, RBI, Boston, MA, USA); 1-aminoadamantane (amantadine, Aldrich, Steinheim, Germany); methyllycaconitine (Sigma); mecamylamine (Sigma); (−)-nicotine (Sigma).

2.5. Statistical analysis

All results are shown as mean ± S.E.M. In case of the maximal electroshock seizure test, the aim was to obtain

ED_{50}'s for all parameters scored (anticonvulsant activity and motor side effects) with use of the test for quantal dose responses (Litchfield and Wilcoxon, 1949). Division of the ED_{50} for side effects on motor function in the rotarod or traction reflex tests by the ED_{50} for antagonism of electroshock convulsions was used as a therapeutic index (TI). Statistical significance of seizure data in the kindling model was calculated by the Wilcoxon signed rank test for paired replicates.

3. Results

3.1. Electrophysiology

The five novel amino-alkyl-cyclohexanes MRZ 2/579, MRZ 2/621, MRZ 2/625, MRZ 2/642, MRZ 2/705, and, for comparison, amantadine, memantine and MK-801, have been tested for their potency on NMDA receptors and nicotinic acetylcholine receptors in patch clamp experiments. Out of them, MRZ 2/621, 2/625 and 2/642 were most selective toward nicotinic acetylcholine receptors (43–142 fold) vs. NMDA receptors (Table 1). On the other hand, MK-801, MRZ 2/579 (neramexane) and memantine were the most selective for NMDA receptors (Table 1). Examples of typical nicotinic and NMDA responses and their antagonism by MRZ 2/705 are shown in Figs. 2 and 3. For the nicotinic responses shown in Fig. 2, the rapid inactivation of these currents indicates that they are mediated via α7 nicotinic acetylcholine receptors (Albuquerque et al., 1995). For comparison with the novel compounds, published data for mecamylamine and methyllycaconitine are shown in Table 1.

3.2. Electroshock- and nicotine-induced convulsions in mice

Except methyllycaconitine, all tested agents inhibited electrically induced convulsions, however with marked differences in potency (Table 1). Among the seven agents which preferentially block nicotinic receptors, MRZ 2/642 and MRZ 2/705 were the most potent compounds, clearly surpassing the potency of mecamylamine. However, the NMDA preferring antagonists MK-801, MRZ 2/579, and memantine were even more potent in the maximal electroshock seizure test than nicotinic acetylcholine receptor preferring antagonists.

An additional injection of a subconvulsant dose of nicotine (2 mg/kg, s.c.) shortly before the maximal electroshock seizure test increased the ED_{50}'s of all agents 2–3-fold, even of those agents that are selective NMDA receptor antagonists such as MK-801 or memantine (Table 1). In some cases, mortality was observed (MRZ 2/625) so that ED_{50} values could not be obtained. Methyllycaconitine has not been tested because of mortality seen in the maximal electroshock seizure test even without nicotine (Table 1).

Convulsions induced by nicotine (8 mg/kg, s.c.) were blocked by mecamylamine and, more potently, by methyllycaconitine (Table 1). The novel nicotinic acetylcholine receptor antagonists MRZ 2/621, 2/642 and 2/705 were

Table 1
Comparison of potency of selected agents as nicotinic acetylcholine receptor (nAChR) and NMDA receptor antagonists in vitro using patch clamp (mean ± S.E.M., $N=3$) in hippocampal neurons

Agent	PC IC_{50} nicotine μM	PC IC_{50} NMDA μM	Selectivity for nAChR receptors	MES test ED_{50} mg/kg i.p.	MES + nicotine ED_{50} mg/kg i.p.	ED_{50} shift by nicotine	Nicotine-induced convulsions ED_{50} mg/kg i.p.	Selectivity for nicotine vs. MES
Mecamylamine	1.8[a]	5[b]	2.7	38.6 (28.4–52.3)	>40	>1	32.6 (61.6–17.2)	1.2
Methyllycaconitine	0.001[c]	>10[d]	10,000	>20 (M)[e]	NT	?	10.9 (14.1–8.5)	>1.8
Amantadine	6.5 ± 0.55	80 ± 10.4[f]	12	184 (122–279)[f]	>250	>1.4	18.3 (25.2–13.3)	10.2
Memantine	12.3 ± 1.25	1.1 ± 0.09	0.09	6.9 (5.4–8.8)[f]	11.68 (9.0–15.1)	1.7	6.1 (4.6–7.8)	1.13
(+)-MK-801	1–15[a,g]	0.14 ± 0.1[f]	<0.14	0.16 (0.13–0.31)[f]	0.59 (0.5–0.7)	3.0	0.25 (0.16–0.39)	0.64
MRZ 2/579	>30	1.3 ± 0.2[f]	<0.04	3.6 (2.2–6.1)[f]	10.97 (9.9–12.1)	3.7	5.61 (3.9–8.1)	0.64
MRZ 2/621	0.65 ± 0.07	92.4 ± 19.50[f]	142	36.9 (22.6–60.3)[f]	70.2 (61.6–80.0)	1.9	3.85 (2.0–7.5)	9.6
MRZ 2/625	3.3 ± 0.62	244 ± 40.5[f]	74	129 (42.5–396)[f]	>130 (M)[h]	>1.5	>130	<1
MRZ 2/642	1.0 ± 0.06	42.5 ± 6.5[f]	43	8.0 (5.1–12.7)[f]	25.68 (18.0–36.7)	3.2	2.46 (1.1–5.6)	3.32
MRZ 2/705	1.3 ± 0.13	21.0 ± 1.3	16	9.5 (4.3–21.1)	32.12 (21.9–47.0)	3.4	5.98 (2.8–12.9)	1.59

Also their potency as antagonists of electroshock and nicotine-induced convulsions is given. For a description of electrophysiology and quantal ED_{50} with confidence limits see Section 2. Abbreviations: MES, maximal electroshock seizure.
NT, not tested.
[a] Briggs and McKenna (1996).
[b] Reynolds and Miller (1988).
[c] Ward et al. (1990).
[d] Rao et al. (1997).
[e] At 20 mg/kg, four out of five mice died 15 min after application (before the maximal electroshock seizure test).
[f] Parsons et al. (1999).
[g] Amador and Dani (1991).
[h] At 130 mg/kg, one mouse died before and four died after nicotine administration (before the maximal electroshock seizure test).

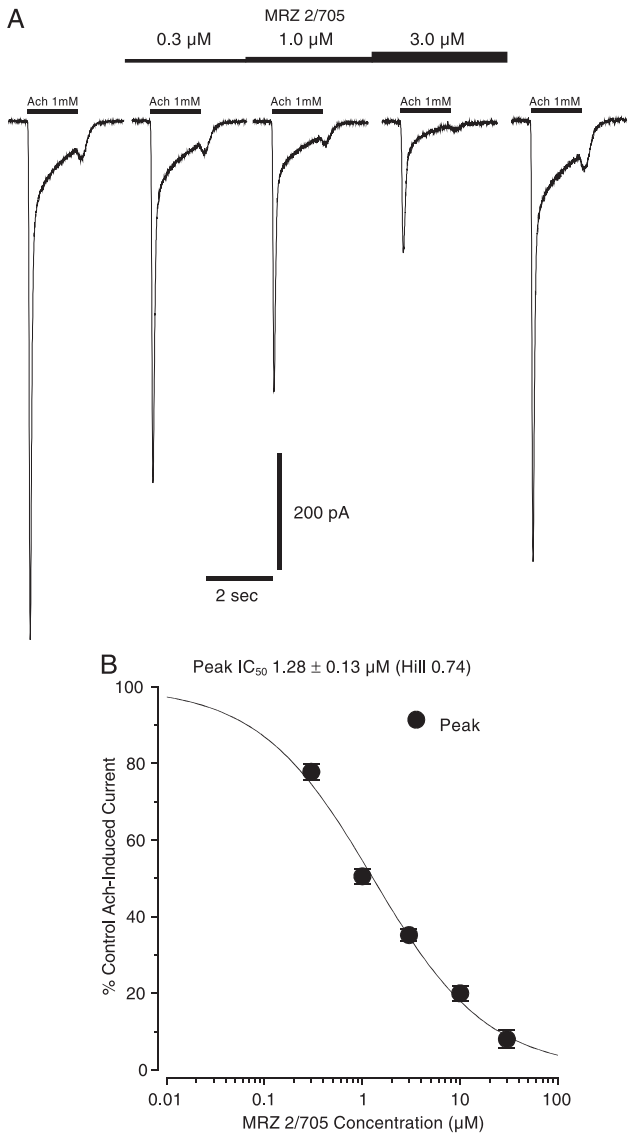

Fig. 2. Concentration-dependence of the blockade of neuronal nicotinic receptors by MRZ 2/705 in cultured hippocampal neurones. Acetylcholine (ACh; 1 mM) was applied for 2 s every 30 s in the continuous presence of various concentrations of MRZ 2/705 (0.3–30 μM) at a constant membrane potential of −70 mV. Even though ACh stimulates both nicotinic Ach receptors and muscarinic ACh receptors, a role of muscarinic receptors in the measured responses to ACh is unlikely, because (1) ACh responses could be blocked by 100% with selective nicotinic antagonists, and (2) activation of metabotropic ACh receptors does not induce a membrane current per se, but rather can influence other voltage-gated channels which were not active in the cells studied. (A) Original data for a single hippocampal neurone-ACh was applied as indicated by the bars. The left and right panels show control and recovery responses, respectively. The middle three panels show equilibrium responses in the continuous presence of MRZ 2/705 (0.3–3 μM), respectively. (B) Peak ACh current responses were normalised to control levels and plotted as means (± S.E.M.) against MRZ 2/705 concentration. Estimation of IC_{50} s and curve fitting were made according to the four-parameter logistic equation (GraFit, Erithacus Software).

more potent to block nicotine-induced seizures than mecamylamine or methyllycaconitine, while MRZ 2/625 was not effective up to 130 mg/kg, possibly because of its relative low potency at nicotinic acetylcholine receptors (Table 1). The NMDA receptor antagonist MK-801 was the most potent anticonvulsant against nicotine, while the NMDA receptor antagonists memantine and MRZ 2/579 showed potencies comparable to those of the nicotinic acetylcholine receptor antagonists. When anticonvulsant ED_{50}'s against electroshock-and nicotine-induced seizures were compared, amantadine and MRZ 2/621 showed the highest selectivity for antagonism of nicotine-induced seizures, followed by MRZ 2/642 and methyllycaconitine (Table 1).

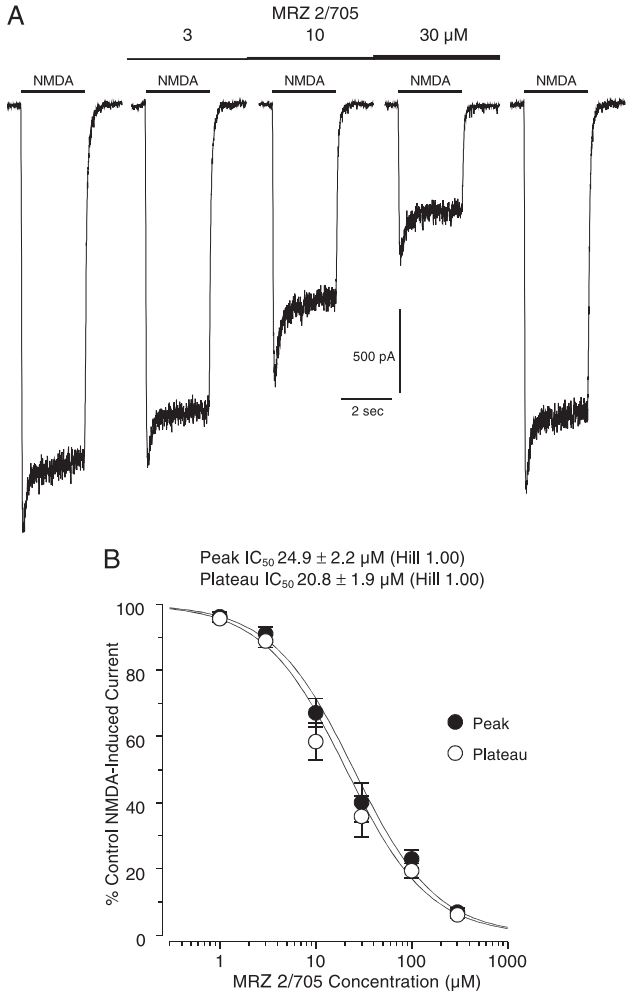

Fig. 3. Concentration-dependence of the blockade of NMDA receptors by MRZ 2/705 in cultured hippocampal neurones. NMDA (200 μM) was applied for 2.5 s every 30 s in the continuous presence of glycine (1 μM) and various concentrations of MRZ 2/705 (1–300 μM) at a constant membrane potential of −70 mV. (A) Original data for a single hippocampal neurone - NMDA was applied as indicated by the bars. The left and right panels show control and recovery responses respectively. The middle three panels show equilibrium responses in the continuous presence of MRZ 2/705 (3–30 μM), respectively. (B) Peak and plateau NMDA current responses were normalised to control levels and plotted as means (± S.E.M.) against MRZ 2/705 concentration. Estimation of IC_{50} s and curve fitting were made according to the four-parameter logistic equation (GraFit, Erithacus Software).

3.3. Adverse effects on motor function in mice

In order to exclude that the anticonvulsant effects of nicotinic acetylcholine receptor and NMDA receptor antagonists in the maximal electroshock seizure test were only secondary to a myorelaxant effect, all agents were tested in the rotarod and traction reflex tests. As shown in Table 2, the nicotinic antagonists mecamylamine and methyllycaconitine had a ''therapeutic index'' (TI) of less than 1, demonstrating that their anticonvulsant doses in the maximal electroshock seizure test was in the dose range inducing motor impairment. In contrast, the nicotinic antagonists MRZ 2/642 and MRZ 2/705 had TI values of 3 or above, showing that the anticonvulsant doses of these compounds were clearly separated from doses inducing motor impairment. A favorable TI was also reached by the NMDA receptor antagonist MRZ 2/579.

3.4. Kindling-induced convulsions in rats

Three of the novel agents, MRZ 2/625, MRZ 2/642, and MRZ 2/705, with the highest selectivity toward nicotinic acetylcholine receptors were chosen for experiments in amygdala-kindled rats. MRZ 2/625 appeared to be more potent against kindled seizures than against generalized tonic seizures in the maximal electroshock seizure test. While the ED_{50} of MRZ 2/625 in the maximal electroshock seizure test was 130 mg/kg (Table 1), significant effects on kindled seizures were seen at 10 and 30 mg/kg (Fig. 4). At 30 mg/kg, the drug significantly increased afterdischarge threshold by 70% and significantly decreased seizure severity, seizure duration, and afterdischarge duration recorded at afterdischarge threshold currents. The only adverse effects in this dose range were minor signs of sedation and ataxia (scores of 1–2 in individual rats), but all rats passed the rotarod test at doses of 3, 10, or 30 mg/kg. Body temperature was not affected by MRZ 2/625.

In contrast to MRZ 2/625, MRZ 2/642 was less efficacious in kindled rats than in the maximal electroshock seizure test in mice. When evaluated at doses of 5, 10, or 30 in kindled rats, the only significant anticonvulsant effect was a decrease in seizure duration at 30 mg/kg, while afterdischarge threshold or seizure severity were not changed (Fig. 5). When the dose of MRZ 2/642 was increased to 50 mg/kg, generalized seizures were induced in a kindled rat before amygdala stimulation. In another kindled rat, the afterdischarge threshold was lowered after 50 mg/kg, and the seizure recorded at afterdischarge threshold current did not terminate spontaneously but had to be interrupted by diazepam. Because of these proconvulsant effects, no further experiments were performed with this dose. In the dose range of 5–30 mg/kg, only slight sedation and ataxia (scores of 1–2 in individual rats) were observed, and all rats passed the rotarod test. Body temperature was not affected by MRZ 2/642.

MRZ 2/705 exerted anticonvulsant effects in kindled rats at doses which were also anticonvulsant in the maximal electroshock seizure test in mice. Thus, a dose-dependent, significant increase in focal seizure threshold (afterdischarge threshold) was observed after 10 (50% increase) and 20 mg/kg (95% increase)(Fig. 6). At 20 mg/kg, seizure severity, seizure duration, and afterdischarge duration were significantly reduced (Fig. 6). Only slight sedation and ataxia (scores of 1–2 in individual rats) were observed, and all rats passed the rotarod test. Body temperature was not affected by MRZ 2/705. When MRZ 2/705 was tested in another group of eight kindled rats in order to prove the reproducibility of its effects, no significant anticonvulsant effects were determined after 10 or 20 mg/kg (not illustrated). There was no obvious explanation for the lack of anticonvulsant activity of MRZ 2/705 in this replicate experiment in kindled rats.

For comparison with the novel nicotinic antagonists, mecamylamine was evaluated in kindled rats at doses of 0.1, 0.33, 3.3 and 6.6 mg/kg (Fig. 7). Mecamylamine significantly increased afterdischarge threshold at a dose of 3.3 mg/kg, but there was no clear dose-dependency of this effect, because lower and higher doses did not exert any significant effect on kindled seizures. In the dose range

Table 2
Adverse effects on motor function as determined in the rotarod and traction reflex tests

Agent	Rotarod ED_{50} mg/kg i.p.	Traction test ED_{50} mg/kg i.p.	TI (rotarod/MES)	TI (traction test/MES)
Mecamylamine	32.2 (24.7–41.9)	17.4 (6.9–43.9)	0.83	0.45
Methyllycaconitine	>20	~ 20	?	<1
Amantadine	104 (85.6–129)	96.6 (90.4–103)	0.57	0.53
Memantine	13.65 (9.9–18.8)	6.5 (3.9–10.7)	2	0.94
(+)-MK-801	0.22 (0.20–0.24)	0.14 (0.11–0.19)	1.4	0.88
MRZ 2/579	18.0 (15.9–21.7)	10.7 (7.8–14.6)	5.0	3.0
MRZ 2/621	54.1 (45.2–64.7)	64.9 (37.8–112)	1.5	1.8
MRZ 2/625	159 (39.7–638)	70.5 (33.8–147)	1.2	0.54
MRZ 2/642	44.2 (32.6–60.0)	26.6 (20.2–34.9)	5.5	3.3
MRZ 2/705	36.4 (17.9–73.9)	28.9 (15.4–54.5)	3.8	3.0

ED_{50}'s (with confidence limits) determined in these tests were used to calculate the ''therapeutic index'' (TI) by dividing the ED_{50} in the motor test by the ED_{50} in the maximal electroshock seizure (MES) test.

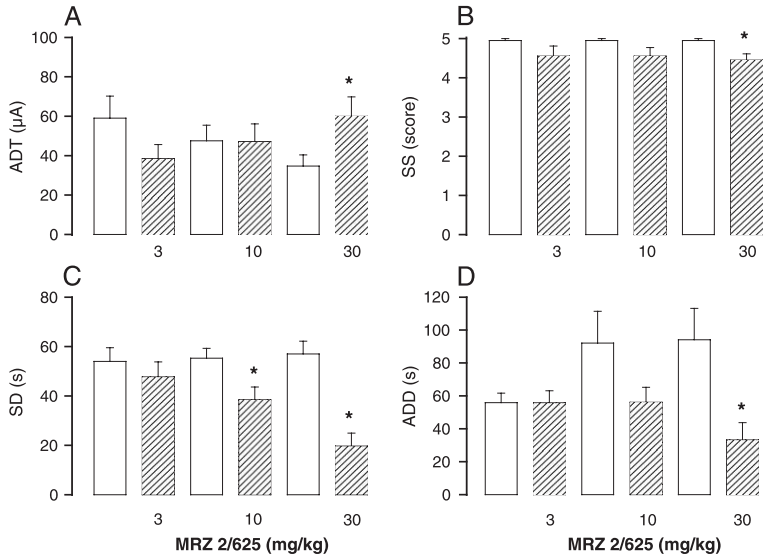

Fig. 4. Anticonvulsant effects of MRZ 2/625 in fully kindled rats. The figure shows the seizure threshold (afterdischarge threshold, ADT; panel A) determined by electrical stimulation of the kindled amygdala with a staircase method, and seizure parameters (panel B: seizure severity [SS]; panel C: seizure duration [SD]; panel D: afterdischarge duration [ADD]) determined at afterdischarge threshold current. Open bars are from vehicle control experiments, while dashed bars are from drug experiments, carried out 2–3 days after the vehicle control in the same group of rats. Vehicle or drug were injected i.p. 30 min before afterdischarge threshold determination. Doses (mg/kg i.p.) are indicated below the bars. All data are means ± S.E.M. of 10 rats. Significant difference to vehicle control in the same group of rats is indicated by asterisk ($P < 0.05$).

examined, no motor impairment was observed, and all rats passed the rotarod test. Furthermore, mecamylamine did not exert any effects on body temperature. The only obvious adverse effect was active (stereotypic) closing of eyes, which was observed after 3.3 and 6.6 mg/kg.

In order to examine whether a combination of anti-nicotinic and anti-NMDA effects leads to anticonvulsant effects in the kindling model, low doses of mecamylamine (1 mg/kg) and MK-801 (0.03 or 0.1 mg/kg) were combined (Fig. 7). MK-801, 0.1 mg/kg, administered alone did not significantly alter afterdischarge threshold, but moderately decreased seizure severity, seizure duration, and afterdischarge duration (Fig. 7). These effects were not potentiated by combined administration with mecamylamine, but, in contrast, no significant anticonvulsant effects were seen with the combination, independent of whether MK-801 was administered at 0.1 or 0.03 mg/kg. While no rotarod failures or behavioral adverse effects were seen with the combination of mecamylamine and 0.03 mg/kg MK-801, marked ataxia (average score of about 4) and 100% rotarod failures were seen both after 0.1 mg/kg MK-801 alone and in combination with mecamylamine. Furthermore, stereotyped behaviors (head weaving, hyperlocomotion) were observed after the higher dose of MK-801. The body

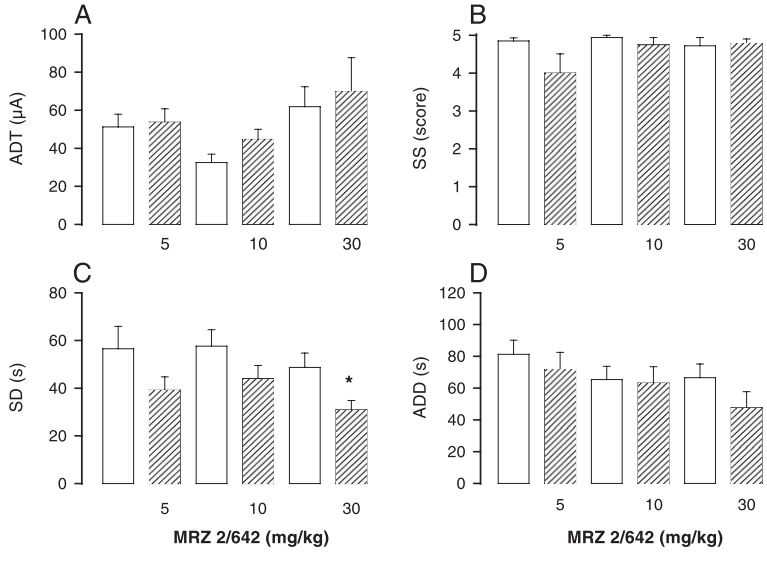

Fig. 5. Anticonvulsant effects of MRZ 2/642 in fully kindled rats. Data are means ± S.E.M. of nine rats. For further details see legend to Fig. 4.

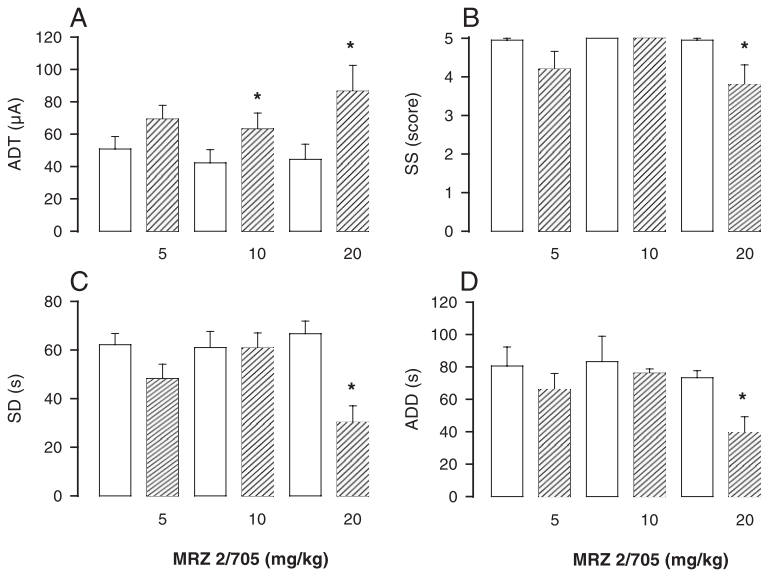

Fig. 6. Anticonvulsant effects of MRZ 2/705 in fully kindled rats. Data are means ± S.E.M. of nine rats. For further details see legend to Fig. 4.

temperature was not altered after MK-801 alone or in combination with mecamylamine.

In additional experiments, different doses of nicotine were i.p. administered in kindled rats in order to evaluate whether kindling changed the convulsant activity of nicotine. At a low dose of 0.1 mg/kg, nicotine induced a decrease in afterdischarge threshold of 60% and 15% in the two kindled rats which received this dosage, but seizure parameters recorded at afterdischarge threshold were not altered. When the dose was increased to 0.2 mg/kg in eight kindled rats, no significant alterations in afterdischarge threshold or seizure parameters recorded at afterdischarge threshold were determined 10 min after injection of nicotine (not illustrated). Adverse effects of nicotine at this dose included moderate ataxia (average score of 2.5), increased heart rate, increased respiratory frequency, and in individual animals also flat body posture. One rat exhibited myoclonic twitches at stimulations below afterdischarge threshold current. Body temperature was not affected by 0.2 mg/kg nicotine. Administration of 1 mg/kg nicotine in two kindled rats resulted in violent generalized seizures and respiratory distress starting about two minutes after injection. The seizure activity was disrupted using diazepam 5 min after seizure onset. In a naive, non-kindled rat, the same dosage

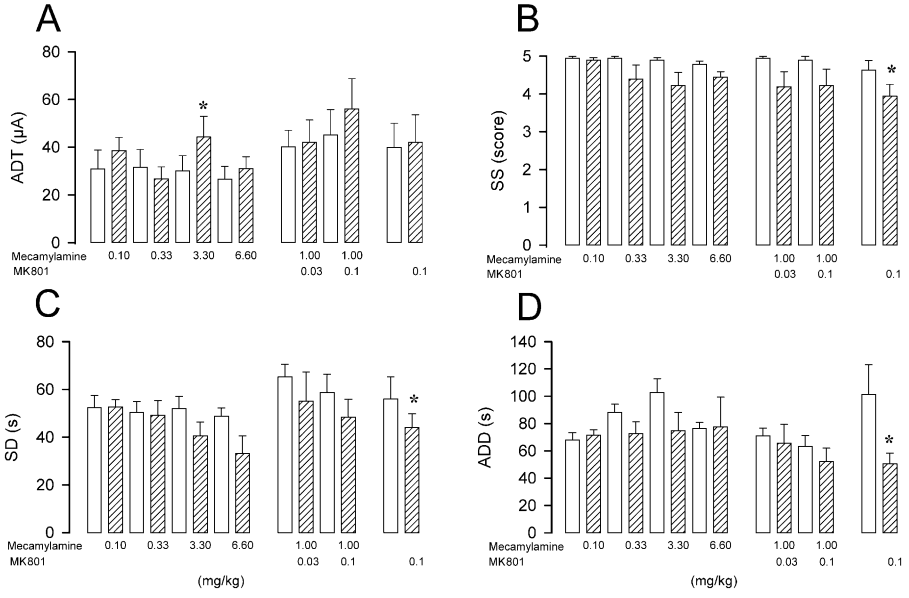

Fig. 7. Effects of mecamylamine and MK-801 in fully kindled rats. Both drugs were either given alone or in combination at the doses indicated below the bars. For the combination experiments, MK-801 was given 60 min and mecamylamine 30 min before amygdala stimulation. Data are means ± S.E.M. of eight to nine rats. For further details, see legend to Fig. 4.

induced vocalization, strong hyperexcitability and hyperlocomotion as well as respiratory distress but no seizures. Following i.p. administration of 0.5 mg/kg nicotine, another naive, non-kindled rat showed sedation, slight difficulties with respiration and signs of medium ataxia. Because of the effects on respiratory function, experiments with such doses of nicotine were not continued in kindled or non-kindled rats.

4. Discussion

The maximal electroshock seizure test in mice is the most widely used animal model for anticonvulsant drug testing (Löscher; 1999). The endpoint in this test is tonic hind limb extension, and the test is thought to be a predictive model for generalized tonic–clonic seizures. The maximal electroshock seizure test is generally considered to indicate a drug's ability to prevent seizure spread (Löscher, 1999). The maximal electroshock seizure test is particularly sensitive to drugs blocking Na^+ channels, so that it is not surprising that almost all antiepileptic drugs, which were initially detected by this test, act, at least in part, via this mechanism (Meldrum, 1997). Furthermore, NMDA receptor antagonists are very potent in the maximal electroshock seizure test (Löscher and Rogawski, 2002), while, to our knowledge, nicotinic acetylcholine receptor antagonists have not been evaluated previously in this model.

In the present study, except methyllycaconitine, all preferential nicotinic acetylcholine receptor antagonists (mecamylamine and the novel agents MRZ 2/621, MRZ 2/625, MRZ 2/642 and MRZ 2/705) inhibited generalized tonic seizures in the maximal electroshock seizure model in mice. The anticonvulsant ED_{50} was shifted to the right by injection of subthreshold dose of nicotine, suggesting the contribution of nicotinic acetylcholine receptor mechanisms. Consistent with previous studies (Löscher and Rogawski, 2002), maximal electroshock seizures were also inhibited by selective NMDA receptor antagonists such as memantine or (+)-MK-801, but, surprisingly, the shift in ED_{50} by nicotine was quite similar for both preferential NMDA and nicotinic acetylcholine receptor antagonists.

As reported previously (Damaj et al., 1999), nicotine-induced convulsions were potently blocked by nicotinic acetylcholine receptor antagonists, such as mecamylamine and methyllycaconitine , and NMDA receptor antagonists such as (+)-MK-801. Furthermore, except MRZ 2/625, the novel amino-alkyl-cyclohexane derivatives were quite potent to block nicotine-induced seizures. Apart from two agents (amantadine, and MRZ 2/621), the selectivity for nicotine-induced seizures vs. maximal electroshock seizures ranged from 0.64 (MK-801) to 3.32 (MRZ 2/642), while selectivity for nicotinic acetylcholine receptors showed over 100,000-fold difference (e.g. methyllycaconitine vs. MRZ 2/579). This could indicate, that in both types of seizures there is some contribution of nicotinic acetylcholine receptor and NMDA receptor related mechanisms. It could be suggested that activation of NMDA receptors play a permissive role while that of nicotinic acetylcholine receptors is more of modulatory nature, i.e. it is sufficient to block the first but not second type to get anticonvulsant effect.

The role of nicotinic acetylcholine receptor subtypes in mediating the convulsant effects of nicotine has been the subject of a number of recent studies (Stitzel et al., 1998, 2000 Damaj et al., 1999; Broide et al., 2002; Franceschini et al., 2002; Gil et al., 2002). The two major subtypes of functional nicotinic acetylcholine receptors in the brain are formed by the heteromeric assembly of $\alpha4\beta2$ subunits (the high affinity nicotine-binding sites), which are relatively abundant in the brain, and the homomeric assembly of $\alpha7$ subunits (Jones et al., 1999). Nicotine binds to the $\alpha4\beta2$ nicotinic acetylcholine receptor with high affinity, whereas the $\alpha7$ nicotinic acetylcholine receptor is 1000-fold less sensitive. Based on experiments with subtype-selective antagonists, the seizures induced by high doses of nicotine are thought to be predominantly mediated by the $\alpha7$-receptor subtype (Damaj et al., 1999). In line with this assumption, in the present experiments the $\alpha7$-selective competitive nicotinic acetylcholine receptor antagonist methyllycaconitine was three times more potent to block nicotine-induced seizures than the archetypical non-competitive nicotinic acetylcholine receptor antagonist mecamylamine, which blocks $\alpha4\beta2$ nicotinic acetylcholine receptors more potently than the $\alpha7$ subtype (Chavez-Noriega et al., 1997). However, more recent mutant mouse studies on the role of $\alpha7$ nicotinic acetylcholine receptors in nicotine-induced seizures have yielded equivocal findings. Mutant mice with an increased function of $\alpha7$ nicotinic acetylcholine receptors showed increased sensitivity to nicotine-induced seizures, which would substantiate an important role of this nicotinic acetylcholine receptor subtype in the mechanisms underlying the convulsive properties of nicotine (Broide et al., 2002; Gil et al., 2002). However, surprisingly mice lacking the $\alpha7$ nicotinic acetylcholine receptor subunit were not less sensitive to the convulsive effects of nicotine, indicating that $\alpha7$ may not be necessary for the mechanisms underlying nicotine-induced seizures (Franceschini et al., 2002).

In contrast to mecamylamine, methyllycaconitine did not block seizures in the maximal electroshock seizure test, indicating that this model is insensitive to manipulation of $\alpha7$ nicotinic acetylcholine receptors. The nicotinic acetylcholine receptor subtype selectivity, if any, of the novel amino-alkyl-cyclohexane series of nicotinic acetylcholine receptor antagonists is not known as yet, but certain discrepancies between agents used in the present experiments may partially result from a different receptor subtype selectivity.

The kindling model in rats is a widely used chronic model of temporal lobe epilepsy with complex-partial seizures (Sato et al., 1990; Löscher, 1999). The term ''kindling'' refers to a phenomenon in which periodic

application of an initially subconvulsive electrical stimulus to a limbic brain region such as the amygdala results in the progressive evolution of persistent seizure susceptibility. Once this susceptibility is fully developed, animals are said to be fully kindled. In such fully kindled rats, each stimulation produces a focal seizure with secondary generalization to clonic seizures. When the model is used in the way done in the present study, determination of afterdischarge threshold in fully kindled rats allows to identify drug effects on seizure threshold in the focus (the amygdala), while determination of severity and duration of seizures recorded at afterdischarge threshold currents is a measure of drug effects on seizure propagation from the focus (Löscher, 1999). Evidence from a variety of approaches strongly indicates that muscarinic cholinergic neurotransmission contributes to electrical kindling in the rat (Burchfiel et al., 1979; Peterson and Albertson, 1982; Bianchi and Beani, 1998; Cain, 1989), but little is known about the role of nicotinic cholinergic transmission. When mecamylamine, 10 mg/kg, was injected prior to each amygdala stimulation during kindling development in rats, kindling rate was not different from controls (Meyerhoff and Bates, 1985). However, when mecamylamine was combined with atropine, kindling development was significantly suppressed (Meyerhoff and Bates, 1985). To our knowledge, effects of nicotinic acetylcholine receptor antagonists have not been studied yet in fully kindled rats.

In the present experiments, mecamylamine significantly increased the focal seizure threshold (afterdischarge threshold) significantly at a dose of 3.3 mg/kg, which is within the dose range to block nicotinic acetylcholine receptors in behavioral studies in rats. However, this anticonvulsant effect was lost at higher doses. An increase in afterdischarge threshold was also seen after the novel nicotinic acetylcholine receptor antagonists MRZ 2/625 and MRZ 2/705, but with the latter agent this effect could not be replicated in another group of kindled rats. MRZ 2/642, which was as potent as MRZ 2/705 in the maximal electroshock seizure test in mice, did not exert any significant effects on afterdischarge threshold in kindled rats. All three agents significantly decreased seizure severity or seizure duration or both, indicating an effect on seizure propagation from the kindled amygdala. At a high dose, MRZ 2/642 induced seizures in kindled rats. Taken together, the anticonvulsant effects of nicotinic acetylcholine receptor antagonists in fully kindled rats appeared to be less robust than their anticonvulsant effect in the maximal electroshock seizure test in mice.

We also examined whether kindling results in an enhanced susceptibility to the proconvulsant effect of nicotine. Low doses of nicotine induced severe seizures in kindled rats, and preliminary data indicate that the seizure threshold to nicotine is lower in kindled compared with nonkindled animals. The chronic brain alterations associated with kindling are known to alter markedly the pharmacology of several agents (Adamec, 1990; Löscher, 1999), and a lowered seizure threshold to several convulsants has been reported previously in kindled rats (Peterson and Albertson, 1982).

Provided that brain nicotinic receptors play a role in the generation or propagation of epileptic seizures, drugs with mixed anti-nicotinic and anti-NMDA properties may form a novel class of antiepileptic drugs. NMDA receptor antagonists are potent anticonvulsant drugs, but with most agents the anticonvulsant activity is not sufficiently separated from adverse effects, e.g. impairment of motor function (Löscher, 1998; Löscher and Rogawski, 2002). Bearing in mind the good clinical tolerability of the NMDA receptor antagonist memantine (Parsons et al., 1998) and interest to develop new substances with similar characteristics by retaining active groups of memantine moiety and their three-dimensional relationship, chemically new derivatives of aminoalkyl-cyclohexanes were developed (Parsons et al., 1999, 2000; Jirgensons et al., 2000). Out of them, MRZ 2/579 (neramexane) seemed to have features most close to that of memantine and recently entered phase II of clinical trials. However, apart from selective NMDA receptor antagonists such as MRZ 2/579, several blockers of nicotinic acetylcholine receptors have been detected with different degree of selectivity vs. NMDA receptors. Some of these agents, which were tested in the present study, showed pronounced anticonvulsant activity against maximal electroshock seizures in mice and the potency was considerable higher than could be predicted taking into account NMDA receptor related mechanisms only. Furthermore, some of these agents had a better therapeutic index than selective NMDA receptor antagonists. Because this could indicate that a combination of nicotinic acetylcholine receptor and NMDA receptor antagonistic actions in a drug provides interesting features for treatment of seizures, we directly addressed this possibility by combined administration of mecamylamine and (+)-MK-801 in kindled rats. The results were not promising, because no significant anticonvulsant effect was produced by combining low doses of the two drugs. These data suggest possible use of agents having combined NMDA receptor and nicotinic acetylcholine receptor antagonistic properties for treatment of generalized seizures but rather not for complex-partial seizures.

In conclusion, the present data indicate that NMDA receptor antagonists having also blocking action at nicotinic acetylcholine receptors are more potent anticonvulsants in the maximal electroshock seizure test in mice, while less clear cut effects are seen in kindled rats. The anticonvulsant effects of nicotinic acetylcholine receptor antagonists and the fact that the nicotinic acetylcholine receptor agonist nicotine and other nicotinic acetylcholine receptor agonists induce seizures in different species, including man (Taylor, 1996) substantiate the suggestion from studies in familal epilepsies that a gain of function of nicotinic acetylcholine receptors may be one pathogenetic mechanism that causes epileptic seizures (Bertrand et al., 2002). Determining the precise role of pre- and postsynaptic nicotinic acetylcholine receptors in generation and propagation of seizures continues to be an

important and novel area of research. Apart from receptor subtype, the receptor localization may determine the functional consequences of alterations in nicotinic acetylcholine receptor functions. This, however, would probably complicate a development of safe and effective antiepileptic drugs based on nicotinic acetylcholine receptor mechanism.

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