Peripherally acting NMDA receptor/glycine$_B$ site receptor antagonists inhibit morphine tolerance

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Abstract

The present study focused on the role of peripheral ionotropic $N$-methyl-$d$-aspartate (NMDA) receptors in the development of tolerance to morphine-induced antinociception. An initial experiment revealed that NMDA channel blocker memantine, and NMDA receptor/glycine$_B$ site antagonist MRZ 2/576 inhibited maximal electroshock-induced convulsions (MES) in female NMR mice with respective potency of 5.93 and 20.8 mg/kg, while other NMDA receptor/glycine$_B$ site antagonists MRZ 2/596 and MDL 105,519 were ineffective, supporting lack of CNS activity of the latter two agents. This observation was also supported by blood–brain barrier experiments in vitro. In male Swiss mice, morphine (10 mg/kg) given for 6 days twice a day (b.i.d.) produced tolerance to its antinociceptive effects in the tail-flick test. The NMDA receptor/glycine$_B$ site antagonists, MRZ 2/576 at 0.03, 0.1, 0.3 mg/kg and MRZ 2/596 at 0.1, 0.3, 3 and 10 mg/kg attenuated the development of morphine tolerance. Similarly, in male C57/Bl mice, morphine (10 mg/kg) given for 6 days b.i.d. produced tolerance to its antinociceptive effects in the tail-flick test. Like in Swiss mice, in C57/Bl mice morphine tolerance was attenuated by both MRZ 2/576 and MRZ 2/596. Another NMDA receptor/glycine$_B$ site receptor antagonist, MDL 105,519 (that very weakly penetrates to the central nervous system) also inhibited morphine tolerance at the dose of 1 but not 0.1 mg/kg. Moreover, both naloxone hydrochloride (5 and 50 mg/kg) and centrally inactive naloxone methiodide (50 mg/kg) inhibited morphine tolerance suggesting the involvement of peripheral opioid receptors in this phenomenon. The present data suggest that blockade of NMDA receptor/glycine$_B$ sites in the periphery may attenuate tolerance to the antinociceptive effects of morphine.

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Keywords: Antinociception; Pain; Glutamate; NMDA receptor/glycine$_B$ site antagonist; Blood–brain barrier

1. Introduction

Over the last decade research has provided compelling evidence that glutamate receptors are crucially involved in phenomena related to opioid tolerance (see Mao, 1999; Price et al., 2000 for reviews). Antagonists of the ionotropic $N$-methyl-$d$-aspartate (NMDA) receptor complex, including memantine, the moderate affinity and highly voltage-dependent clinically used NMDA channel blocker (Parsons et al., 1999) inhibit the development of morphine tolerance (Trujillo and Akil, 1999).
is a selective high affinity antagonist at the NMDA receptor/glycineB site and has recently been introduced as a commercially available radioligand (Amersham Biosciences, Freiburg, Germany) (Baron et al., 1996; Baron et al., 1997). Non-specific binding was defined by the addition of unlabeled glycine at 100 μM. Incubations were terminated using a Millipore filter system (Millipore, Schwabach Germany). The samples, all in duplicate, were rinsed three times with 2.5 ml ice-cold assay buffer over glass fibre filters (Schleicher and Schuell, Dassel, Germany) under a constant vacuum. Filtration was performed as rapidly as possible (max 2 s). Following separation and rinse, the filters were placed into scintillation liquid (5 ml; Ultima Gold) and radioactivity was determined with a liquid scintillation counter (both Packard BioScience, Dreieich, Germany).

2.1.2. Patch clamp

Patch clamp recordings were made from rat hippocampal neurons, after 12–15 days in vitro, with polished glass electrodes (3–5 MΩ) in the whole cell mode at room temperature (20–22 °C) with the aid of an EPC-7 amplifier (HEKA, Lambrecht, Germany) – detailed methods described in Parsons et al. (1999).

2.1.3. BBB permeability studies

An in vitro model of the BBB has been established as a first screen for BBB permeability.

2.1.3.1. Preparation and cultivation of BBCEC. Bovine brain capillary endothelial cells (BBCEC) were isolated from brains, purified, and cultured according to Meresse et al. (1989). Briefly, after mechanical homogenization microvessels were seeded onto dishes coated with an extracellular matrix secreted by bovine corneal endothelial cells (Gospodarowicz et al., 1976). Pure colonies of endothelial cells were seeded onto gelatin-coated dishes (Corning Costar, Bodenheim, Germany) in the presence of Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco Invitrogen GmbH, Karlsruhe, Germany) supplemented with 20% calf serum (HyClone, Utah, USA), 2 mM glutamine, 50 μg/ml of gentamycin (Biochrom, Berlin, Germany), and bovine fibroblast growth factor (bFGF; Roche, Mannheim, Germany), in the presence of Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco Invitrogen GmbH, Karlsruhe, Germany) containing 10% FCS (HyClone, Utah, 1991; Marek et al., 1991; Popik et al., 2000a) (see Bespalov and Trujillo, 2002 for the recent review).

One obstacle to the introduction of NMDA receptor antagonists into clinical practice is undesired “phencyclidine side-effects” profile, that is centrally mediated (Parsons et al., 1999). In turn, NMDA receptor antagonists which weakly penetrate to the brain might have a more favorable profile. However, such compounds may not be applicable to the inhibition of tolerance to morphine-induced antinociception which is believed to be primarily of central origin (McNally and Westbrook, 1998; McNally, 1999; Ueda and Inoue, 1999). On the other hand, recent data reported by Kolesnikov and colleagues demonstrated that local (topical) application of uncompetitive NMDA receptor antagonists, (+)MK-801 or ketamine, inhibited tolerance to topically applied morphine (Kolesnikov et al., 1996; Kolesnikov and Pasternak, 1999b; Kolesnikov and Pasternak, 1999a), suggesting a peripheral component of antinociceptive morphine tolerance. Since systemic rather than local administration of compounds is more favorable from the therapeutic perspective, the aim of the present study was to investigate whether antagonism of NMDA receptors in the peripheral nervous system (PNS) would inhibit tolerance to the antinociceptive effects of systemically administered morphine. As pharmacological tools we used recently developed NMDA receptor antagonists acting at the NMDA receptor/glycineB site and has recently been introduced as a commercially available radioligand (Amersham Biosciences, Freiburg, Germany) (Baron et al., 1996; Baron et al., 1997). Non-specific binding was defined by the addition of unlabeled glycine at 100 μM. Incubations were terminated using a Millipore filter system (Millipore, Schwabach Germany). The samples, all in duplicate, were rinsed three times with 2.5 ml ice-cold assay buffer over glass fibre filters (Schleicher and Schuell, Dassel, Germany) under a constant vacuum. Filtration was performed as rapidly as possible (max 2 s). Following separation and rinse, the filters were placed into scintillation liquid (5 ml; Ultima Gold) and radioactivity was determined with a liquid scintillation counter (both Packard BioScience, Dreieich, Germany).

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USA), 2 mM glutamine and 50 μg/ml gentamycin (Biochrom, Berlin, Germany).

2.1.3.3. Co-cultivation of BBCEC with rat cortical astrocytes. For co-cultivation the BBCEC were seeded onto a collagen-coated filter (Transwell Col; Corning, Wiesbaden, Germany) and placed into 12-well plates (Corning, Wiesbaden, Germany) containing differentiated astrocytes in BBCEC culture medium.

2.1.3.4. Transport studies. The transport studies were performed between 8 and 12 days of co-cultivation in Krebs Ringer buffer. The amount of substance passing through the endothelial cell layers after 10, 20, and 30 min was quantified in samples taken from the abluminal (brain) side of the filter (performed with 1 min intervals) of each test were considered to exhibit myorelaxation or ataxia, respectively. In case of reaching the criterium on the 1st or 2nd trial, no further trials were performed. These tests were followed 1–2 min later by MES (100 Hz, 0.5 s shock duration, 50 mA shock intensity, 0.9 ms impulse duration, Ugo Basile, Italy). For the traction reflex test, mice were placed with their forepaws on a horizontal rod and were required to place all 4 paws on the wire within 10 s. To test ataxia (motor coordination) mice were placed on rotarod (5 r.p.m.) and were required to remain on the rod for 1 min. Only mice not achieving the criteria in all three repetitions (performed with 1 min intervals) of each test were used.

2.2.1.1. Subjects

2.2.2.1. Subjects. Male Swiss mice (25–30 g, Institute of Pharmacology breeding facility, Krakow, Poland) were group-housed in standard laboratory cages and kept in a temperature-controlled colony room (21 ± 2 °C) with a 12-h light/dark cycle (light on: 07:00). Similarly housed and maintained male C57/Bl mice (25–30 g) were obtained from the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. Commercial food and tap water were available ad libitum. Each experimental group consisted of at least 7 mice per treatment in morphine tolerance experiments and at least 5 mice per treatment in acute morphine antinociception experiment. All mice were used only once.

Morphine tolerance studies were carried out according to the National Institutes of Health Guide for Care
and Use of Laboratory Animals (revised 1996) and were approved by the Institute of Pharmacology PAN Animal Care and Use Bioethics Commission.

The tail-flick tests were performed as described previously (Popik et al., 2000). The assessments of antinociceptive ED$_{50}$ of morphine (test #1 and test #2) were carried out without pretreatment with glutamate and opioid antagonists.

2.2.2.2. Experimental design. The first experiment was carried out on Swiss mice to determine whether the development of tolerance to the antinociceptive effects of morphine could be inhibited by NMDA receptor/glycine$_B$ site antagonists not penetrating (MRZ 2/596) or weakly penetrating (MRZ 2/576) the BBB.

On day 1 the first measurement of morphine antinociceptive potency was performed (test #1), followed by 6 days of b.i.d. morphine injections (10 mg/kg, s.c., 9:00 and 17:30) (Elliott et al., 1994; Popik et al., 2000b). MRZ 2/596 (0.05, 0.1, 0.3, and 1 mg/kg) and MRZ 2/576 (0.01, 0.03, 0.1, 0.3, 1, 3 and 10 mg/kg) were given s.c. 30 min prior to each morphine dose on days 2–7. On day 8 the second measurement of the antinociceptive potency of morphine was performed (test #2). The degree of morphine tolerance was assessed by comparing the antinociceptive potency of morphine (ED$_{50}$) observed in tests #1 and #2.

The subsequent experiments were carried out several months later and due to technical reasons C57/Bl mice were used. In order to confirm results obtained in Swiss mice, the effect of MRZ 2/596 (0.3 mg/kg), MRZ 2/576 (1 mg/kg) on the development of morphine tolerance was tested in C57/Bl mice. To further confirm the usefulness of C57/Bl mice in the present experimental settings, we also used the NMDA channel blocker memantine (2.5 and 7.5 mg/kg) that has been shown previously to inhibit morphine tolerance in Swiss mice (Popik et al., 2000a). Since the effects obtained with Swiss mice were also observed in C57/Bl mice, further experiments were conducted using of C57/Bl mice. To investigate the possibility that the effects of MRZ 2/596 on the development of morphine tolerance were due to an inhibition of morphine’s antinociceptive action (naloxone-like effect), MRZ 2/596 was administered in a single injection of 1 mg/kg s.c. 30 min before single morphine 3 mg/kg s.c. administration. The antinociceptive effects of morphine were investigated 30, 60 and 120 min later. In another control experiment, we investigated if the inhibitory effects of MRZ 2/596 on the development of morphine tolerance could be attributed to an accumulation of this compound. To this end, groups of mice were treated for 7 days twice daily with saline or MRZ 2/596 (1 mg/kg). Twelve hours after the last injection, mice were injected with 3 mg/kg s.c. of morphine and tested in the tail-flick apparatus 30, 60 and 120 min.

The next experiment was carried out to find doses of opioid antagonists to affect acute morphine antinociception. Thus, naloxone hydrochloride (1, 5, 10 and 50 mg/kg) and naloxone methiodide (5, 10 and 50 mg/kg) were administered s.c. 15 min before 10 mg/kg of s.c. morphine (the dose used in chronic experiments) and the tail-flick test was conducted 30 min after morphine injection.

Further, it was determined if (a) another NMDA receptor/glycine$_B$ site antagonist, MDL 105,519, structurally dissimilar from both MRZ 2/576 and MRZ 2/596, and also weakly penetrating the CNS and (b) opioid receptor antagonists, naloxone hydrochloride and its quaternary derivative, naloxone methiodide affect the development of morphine antinociceptive tolerance. MDL 105,519 (0.1 and 1 mg/kg) was given s.c. 30 min prior to each morphine dose. Naloxone hydrochloride (1, 5 and 50 mg/kg) and naloxone methiodide (1, 5 and 50 mg/kg) were given s.c. 15 min prior to each morphine dose during its chronic administration. Doses of morphine and memantine were used based on previous observations (Popik and Skolnick, 1996; Popik et al., 2000b).

2.2.2.3. Data presentation and statistics. Latencies (in s) of the tail-flick responses were converted to %Maximum Possible Effect values [%MPE (Paronis and Holtzman, 1991)], according to the formula: 100([post-injection latency – baseline latency]/[cut-off latency – baseline latency]). %MPE values were used to construct morphine cumulative dose–response curves by non-linear regression; these curves were used to calculate antinociceptive ED$_{50}$ values using GraphPad Prism ver. 3.00 (GraphPad Software, CA, USA) software. The ED$_{50}$ values obtained for tests #1 and #2 were compared among groups, as were the fold shifts (determined by dividing individual test #2 ED$_{50}$ values by the test #1 ED$_{50}$ values) with one-way ANOVAs and post hoc Newman-Keul’s and LSD tests. Data are presented as mean ± S.E.M.

2.2.3. Drugs

MRZ 2/501 (8-chloro-1,4-dioxo-1,2,3,4-tetrahydropyridazino (4,5-b) quinoline), MRZ 2/596 (8-chloro-1,4-dioxo-1,2,3,4-tetrahydropyridazino (4,5-b) quinoline choline salt), MRZ 2/502 (8-chloro-4-hydroxy-1-oxo-1,2-dihydropyridazino (4,5-b) quinoline-5-oxide), and MRZ 2/576 (8-chloro-4-hydroxy-1-oxo-1,2-dihydropyridazino (4,5-b) quinoline-5-oxide choline salt) and memantine (1-amino-3,5-dimethyladamantane) were from Merz Pharmaceuticals GmbH, Frankfurt/M, Germany. Morphine sulphate was obtained from Sigma, Taufkirchen, Germany.

$[^3]$H-morphine was purchased from NEN-Research Products, Köln, Germany, $[^3]$H-MDL 105,519 ((E)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1H-indole-2-
carboxylic acid) and \([^{14}C]\)-sucrose purchased from Amersham Life Science, Freiburg, Germany.

Morphine HCl (morphine tolerance studies) was obtained from Polfa, Kraków, Poland and naloxone hydrochloride, naloxone methiodide, and MDL 105,519 ((E)-3-(2-phenyl-2-carboxyethyl-4,6-dichloro-1H-indole-2-carboxylic acid) from Sigma–Aldrich, USA. All other compounds were obtained from Tocris, U.K. For morphine tolerance experiments morphine, MDL 105,519, memantine, naloxone hydrochloride and naloxone methiodide were dissolved in physiological saline (placebo). MRZ 2/596 and MRZ 2/576 were dissolved in sterile water. All injections were administered in a volume of 10 ml/kg. All NMDA receptor/glycine\(_B\) site antagonist solutions were prepared fresh the day before the experiment started, aliquoted and stored at 4 °C but administered at room temperature. The dose of morphine is expressed as the base, the doses of all other compounds as their respective salts.

3. Results

3.1. In vitro

3.1.1. Receptor binding

In Scatchard analysis \([^3H]\)MDL 105,519 had a \(K_d\) of 6.03 ± 0.14 nM and a \(B_{\text{max}}\) of 4.03 pmol/mg protein. MRZ 2/576 and MRZ 2/596 displaced \([^3H]\)MDL-105,519 binding to rat cortical membranes with \(K_d\) of 126 and 160 nM, respectively (Table 1, \(IC_{50}\) corrected according to the Cheng-Prussoff relationship for 2 nM \([^3H]\)MDL 105,519). MDL-105,519 displaced its own binding with a \(K_i\) of 11.6 nM. Non-specific binding determined with glycine 100 \(\mu\)M was only 15–20% and standard compounds displaced binding to non-specific levels with potencies similar to the literature and Hill coefficients close to unity.

3.1.2. Patch clamp

Steady-state inward current responses of cultured hippocampal neurons to NMDA (200 \(\mu\)M with glycine 1 \(\mu\)M) were antagonized by MDL-105-519 with \(IC_{50}\) of 106 ± 13 nM (Table 1) whereas MRZ 2/576 and MRZ 2/596 with \(IC_{50}\)s of 540 ± 30 and 700 ± 35 nM, respectively, were 5–7-fold less potent (Table 1). When corrected for the affinity of glycine under these conditions (399 nM) all three antagonists had very similar \(K_b\) values to their \(K_d\) assessed in the \([^3H]\)MDL-105,519 binding experiments – \(K_b\): MRZ 2/576 = 154 nM, MRZ 2/596 = 199 nM, MDL 105,519 = 30.3 nM.

3.1.3. BBB permeability studies

As expected, the impermeant marker sucrose showed a low permeability (pe) coefficient of \(2.27 \times 10^{-5}\) cm/s (Fig. 1) in the established BBB model. The lipophylic NMDA channel blocker memantine had good penetration through the in vitro BBB (pe-coefficient 50.32 \(\times 10^{-5}\) cm/s) (Fig. 1). In contrast, the hydrophylic NMDA receptor/glycine\(_B\) site antagonist MDL 105,519 diffused slowly through the endothelial monolayer (pe-coefficient \(2.00 \times 10^{-5}\) cm/s) (Fig. 1).

MRZ 2/576 and its free base MRZ 2/502 showed pe-coefficients of \(2.43 \times 10^{-5}\) and \(25.07 \times 10^{-5}\) cm/s, respectively. The quinoline, MRZ 2/596 and its free base MRZ 2/501 demonstrated pe-coefficients of \(3.60 \times 10^{-5}\) and \(3.33 \times 10^{-5}\) cm/s, respectively (Fig. 1). In the case of MRZ 2/596, the accuracy of the calculated pe-coefficient was limited by the sensitivity of the HPLC-method used. The concentrations of most analytical samples taken

![Fig. 1. In vitro BBB penetration of NMDA receptor/glycine\(_B\) site antagonists in comparison to standards – sucrose poor penetration, memantine good penetration.](image-url)
from the BBCEC monolayers were under the detection limit (100 nM). Therefore the concentration of the
detection limit had to be used for pe-coefficient
calculation which leads to a slightly higher pe-value as
the actual one.

3.1.4. BBB integrity study

BBCEC treated with morphine on the luminal side
(3 μg/ml for 1 h, 3 μg/ml for 6 h, 6 μg/ml for 6 h and
10 μg/ml for 24 h) showed no significant increase in
[14C]-sucrose permeability in comparison to untreated
endothelial cells (data not shown).

3.2. In vivo

3.2.1. Maximal electroshock (MES), traction
reflex and rotarod tests

Memantine and MRZ 2/576 inhibited MES with
ED50s of 5.93 and 20.8 mg/kg, respectively, while both
MRZ 2/596 and MDL 105,519 were ineffective (Table 2).

Memantine disturbed the traction reflex with an
ED50 = 12.5 mg/kg and ataxia was observed with an
ED50 = 14.2 mg/kg. MRZ 2/576 produced myorelax-
atation with an ED50 = 21.1 mg/kg and ataxia with an
ED50 = 22.3 mg/kg. MRZ 2/596 and MDL 105,519
administered even at very high doses did not show any
activity in these tests (Table 2).

All mice treated semi-chronically with MRZ 2/596
(10 mg/kg) for 7 days showed tonic convulsions in the
absence of drug as in control animals (8/8 mice). In
contrast, in mice treated acutely with MRZ 2/576
(20 mg/kg) occurrence of tonic seizures was much lower
(1/8 mice).

3.2.2. Morphine tolerance

The initial experiment carried out on Swiss mice
demonstrated no differences among groups in antinoci-
ceptive morphine ED50 values in test #1. The following
ED50 values (mg/kg) were observed in test #1 for
placebo + placebo: 5.40 ± 0.68; for placebo + mor-
phine: 5.17 ± 0.62; for MRZ 2/596 (at 0.05, 0.1, 0.3,
1 mg/kg + morphine): 5.83 ± 1.97, 4.76 ± 0.70, 5.03 ±
1.36, 4.87 ± 0.95, respectively; and for MRZ 2/576
(0.01, 0.03, 0.1, 0.3, 1, 3, 10 mg/kg) + morphine:
3.43 ± 0.62, 5.10 ± 0.75, 3.95 ± 0.49, 4.99 ± 0.67,
2.88 ± 0.65, 4.53 ± 0.61, 2.99 ± 0.50, respectively
(ANOVA F(12, 158) = 0.92). Six-day b.i.d. treatment
with 10 mg/kg of morphine produced a 3.47-fold
increase in the morphine antinociceptive ED50 values
as determined in test #2. MRZ 2/596 and MRZ 2/576
inhibited morphine antinociceptive tolerance at doses
starting at 0.1 and 0.03 mg/kg, respectively. This was
revealed by smaller differences between tests #1 and #2
(ED50 test #2/ED50 test #1 fold changes, presented in
Fig. 2) in respective groups as compared to control
group that received placebo + morphine. The raw data
of the representative groups are also shown in Fig. 3.

For reasons explained in the methods, all the
subsequent experiments were carried out in C57Bl/mice.
In the acute morphine antinociception experiment,
naloxone hydrochloride at doses of 5, 10 and 50 mg/kg
and naloxone methide at a dose of 50 mg/kg prevented
acute antinociception induced by morphine
(10 mg/kg) (Fig. 4).

Six-day administration of morphine induced a
2.67 ± 0.42 fold change in morphine potency in test

<table>
<thead>
<tr>
<th>Agent</th>
<th>MES ED50 (mg/kg)</th>
<th>Traction ED50 (mg/kg)</th>
<th>Ataxia ED50 (mg/kg)</th>
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<tr>
<td>Memantine</td>
<td>5.93 (3.7–9.5)</td>
<td>12.5 (8.0–19.4)</td>
<td>14.2 (10.2–19.9)</td>
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<tr>
<td>MRZ 2/576</td>
<td>20.8 (15.2–28.4)</td>
<td>21.1 (17.2–25.9)</td>
<td>22.3 (19.9–25.6)</td>
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<tr>
<td>MRZ 2/596</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
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<tr>
<td>MDL 105,519</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
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Values are ED50s in mg/kg (95% confidence limits are shown in parentheses).

Fig. 2. Effects of NMDA receptor/glycineB site antagonists on the
development of morphine antinociceptive tolerance in Swiss mice.
Presented data show fold shifts in morphine antinociceptive ED50
values (mean ± S.E.M.) between test #1 and test #2, before and after
6-day morphine administration (ANOVA F(12, 158) = 4.27, 
p < 0.001). Asterisks indicate a statistically significant difference
toward “Placebo + Morphine” group that received saline and
morphine during the development of morphine tolerance ( * p < 0.05,
** p < 0.01).
#2 (as compared to test #1) that was significantly higher than the placebo treated group (fold change \(1.04 \pm 0.13\)). MRZ 2/596 at 0.3 mg/kg and MRZ 2/596 at 1 mg/kg given prior to each morphine dose inhibited the development of morphine antinociceptive tolerance (fold changes \(1.52 \pm 0.16\) and \(1.44 \pm 0.17\), respectively) (ANOVA \(F(3, 54) = 6.45, p < 0.001\)). This confirms that the phenomenon previously observed in albino Swiss mice can also be demonstrated in C57/Bl mice. Thus, further experiments were continued on C57/Bl mice using the same morphine tolerance paradigm.

In the next set of experiments, there were no differences among C57/Bl mice in the antinociceptive morphine ED\(_{50}\) values in test #1. Test #1 ED\(_{50}\) values (mg/kg) were obtained: for placebo + placebo, 1.92 \(\pm 0.16\); placebo + morphine 1.51 \(\pm 0.13\); memantine (2.5 and 7.5 mg/kg) + morphine 1.40 \(\pm 0.22, 1.59 \pm 0.29\), respectively; MDL 105,519 (0.1, 1 mg/kg) + morphine 1.53 \(\pm 0.22, 1.86 \pm 0.31\), respectively; naloxone hydrochloride (1, 5, 50 mg/kg) + morphine 1.66 \(\pm 0.23, 1.86 \pm 0.26, 2.02 \pm 0.30\), respectively. **Fig. 4.** The effects of naloxone hydrochloride and naloxone methiodide on acute morphine (10 mg/kg)-induced antinociception in the tail-flick test in C57/Bl mice. The opioid receptor antagonists were administered 15 min before morphine and the morphine effect was tested 30 min after its injection (ANOVA \(F(3, 48) = 23.00, p < 0.001\)). Asterisks indicate a statistically significant difference toward “Placebo + Morphine” group that received saline and morphine (\(**p < 0.01, ***(p < 0.001)."

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**Fig. 3.** The figures present morphine dose–response curves obtained before (test #1) and after (test #2) 6-day twice daily morphine administration with MRZ 2/596 (the lowest and the highest dose among administered). The titles describe the chronic treatment between tests #1 and #2. Chronic morphine administration produced right shift of morphine dose–response curve in test #2 (B) as compared to placebo treated group (A). MRZ 2/596 at the dose of 1 mg/kg given prior to each morphine injection prevented the right shift of morphine dose–response curve (D). MRZ 2/596 at 0.05 mg/kg was ineffective (C).
Treatment with 10 mg/kg b.i.d. of morphine produced a 4.55-fold increase in the morphine antinociceptive ED\textsubscript{50} values as determined in test #2. Morphine tolerance was attenuated by memantine at a dose of 7.5 but not at 2.5 mg/kg, and MDL 105,519 at the dose of 1 but not at 0.1 mg/kg (Fig. 5A).

Naloxone hydrochloride prevented the development of morphine antinociceptive tolerance at doses of 5 and 50 but not 1 mg/kg. Naloxone methiodide prevented morphine tolerance at doses of 50 but not 5 and 1 mg/kg (Fig. 5B).

Additionally, the possible acute effect of MRZ 2/596 on morphine-induced antinociceptive activity was investigated. Area Under the Curve calculated on %MPE revealed very similar potency of all three antagonists in both assays. The effective concentrations in vivo will clearly depend on the endogenous levels of glycine and could differ in the CNS and the periphery. Certainly, considerable evidence suggests that the glycine\textsubscript{B} site is not saturated in the CNS in vivo (Danysz and Parsons, 1998).

The antagonism observed with MRZ 2/576 and MRZ 2/596 was typical for moderate affinity NMDA receptor/glycine\textsubscript{B} antagonists, i.e. they induced glycine-sensitive desensitization whereas MDL-105,519 was almost equally effective against peak and plateau – an effect probably related to its higher affinity – see Parsons et al. (1997). Neurochemical studies using radiolabelled MDL 105,519 demonstrate that its brain penetration (plasma vs. brain levels) is low, and the uptake of radioactivity into the brain is in a range of 0.01–0.08% of the injected dose (Opackajuffry et al., 1998). The MES experiment in the present study is in line with this data and also indicates that MRZ 2/596 has negligible CNS effects. Thus, both MRZ 2/596 and MDL 105,519 seem to be devoid of CNS activity, at least at the doses used. This assumption could also be confirmed by the present BBB permeability study where both compounds pass the BBB very slowly as compared with memantine. The effect of morphine on BBB integrity was also investigated as Oishi et al. (1989) reported that morphine treatment affects BBB permeability. The present results indicate that morphine treatment did not affect BBB integrity in vitro since there was no difference in sucrose permeability after incubation with morphine.

In principle, the present data indicate that structurally similar substances with different penetration to the CNS can be discriminated in the BBB in vitro model. Of the glycine\textsubscript{B} antagonists tested, only those with a
NO-substitution could pass the blood–brain barrier (MRZ 2/502 and MRZ 2/576) in contrast to the quinolines (MRZ 2/501 and MRZ 2/596) and MDL 105,519 confirming results of functional studies such as MES. However, it should be stressed that this model can only be regarded as a “first line screen” for CNS penetration since it does not reflect all aspects of governing CNS bioavailability in vivo such as e.g. probenecid sensitive transport out of the brain in the choroid plexus.

The present study revealed that NMDA receptor antagonists acting at the NMDA receptor/ glycineB site with modest (MRZ 2/576) or likely no CNS activity such as MRZ 2/596 and MDL 105,519 inhibited tolerance to the antinoceptive effects of morphine. The effects of the structurally similar moderate affinity agents MRZ 2/576 and MRZ 2/596 were compared to the structurally different high affinity NMDA receptor/ glycineB site antagonist MDL 105,519. All three NMDA receptor/glycineB site antagonists inhibited morphine tolerance suggesting that the inhibition of morphine tolerance by both MRZ 2/576 and MRZ 2/596 is related to their common pharmacodynamic action, i.e. blockade of NMDA receptors at the glycineB site, and not via actions at an unrelated site due to chemical similarity.

The inhibition of morphine tolerance by glycineB antagonists has been well documented. The full glycineB antagonist, ACEA-1328 (20 mg/kg) completely blocks tolerance to morphine-induced antinociception in the tail-flick test in CD-1 mice (Lutfy et al., 1999). Similarly, another antagonists GV 196771A prevented development of tolerance to morphine antinociception in the formalin test in mice (Quartaroli et al., 2001). Even the low intrinsic activity partial agonist, (R, +)-HA966, was able to inhibit morphine tolerance in neuropathic rats (Christensen et al., 2000).

It is certain that the doses of MRZ 2/576 that effectively inhibited morphine tolerance (starting at 0.03 mg/kg) do not reach sufficient concentrations in the brain to block CNS NMDA receptors. Brain microdialysis experiments revealed that only 2% of systemically applied MRZ 2/576 is found in the extracellular fluid in the CNS (Hesselink et al., 1999b). Despite good BBB permeability properties, as described also in our study in the in vitro BBB model, MRZ 2/576 is quickly removed from the brain by probenecid-sensitive transporters (Hesselink et al., 1999b).

Moreover, a number of behavioral studies showed that CNS-related effects of MRZ 2/576 occur at doses much higher than the minimal effective dose in the present report. MRZ 2/576 shows neuroprotective properties at 5 mg/kg (Wenk et al., 1998), anticonvulsant effects at 10 mg/kg (Karcz-Kubicha et al., 1999) and generalization to ethanol cue in drug discrimination test at 5 mg/kg (Bienkowski et al., 1998). In addition, MRZ 2/576 inhibited firing produced by iontophoretic application of NMDA to the dorsal horn spinal neurons with an ED50 = 2.8 mg/kg after intravenous administration (Parsons et al., 1997). In the present study, anticonvulsant, ataxia-producing and traction reflex disturbing ED50 were ~20 mg/kg, which is over 200 times higher than the dose inhibiting morphine tolerance. In contrast, doses of memantine inhibiting MES convulsions and morphine tolerance were very similar and there was only 50% difference in the case of ataxia and traction reflex disturbance. Thus, it seems very unlikely that MRZ 2/576 given s.c. at the dose of 0.03 mg/kg is present in the brain at a concentration sufficient to block NMDA receptors to the degree producing inhibition of morphine tolerance. With regard to MRZ 2/596 and MDL 105,519, these compounds show no CNS-related anti-convulsant activity against maximal electroshock at 50 mg/kg in adult mice (present study). This apparent dissimilarity of the potencies in morphine tolerance and other behavioral measures (ataxia, suppression of convulsions) as well as the lack of parallelism in potencies in various tests (patch clamp and binding studies: MDL 105,519 > MRZ 2/576 = MRZ 2/596; morphine tolerance: MRZ 2/576 > MRZ 2/596 > MDL 105,519) may suggest that different neuronal systems are involved in mediating these effects. At face value, the effectiveness of glycine site NMDA receptor antagonists not penetrating to the brain in inhibiting morphine tolerance appears counterintuitive. However, these findings do not contradict a bulk of earlier findings demonstrating that centrally active NMDA receptor antagonists produce the same effects. This is because different, redundant neuronal targets may mediate interacting effects of various glutamate antagonists.

The importance of peripheral mechanisms for tolerance to the analgesic action of systematically administered morphine has been suggested in the past. Raghavendra and Kulkarni (1999) reported that melatonin reverses the development of tolerance and dependence to morphine and suggested that peripheral benzodiazepine receptors are involved. Possible mechanisms have been suggested by a recent study by Patierno et al. (submitted for publication). Opioid mu receptor endocytosis in enteric neurons induced by abdominal laparotomy was significantly inhibited by NMDA receptor antagonists including MRZ 2/576 and MRZ 2/596 (with similar potency), but not by the AMPA receptor antagonist CNQX. Also mu receptor endocytosis in neurons from tissue exposed to NMDA and from electrically stimulated preparations was pronounced and prevented by NMDA receptor antagonists. It has been suggested that such receptor internalization is evoked by local release of endogenous opioids and that this is modulated by NMDA receptors. Such a mechanism could play a role in the peripheral component of tolerance to the analgesic effects of morphine.
It was also previously reported that systemic administration of morphine for 4 days (10 mg/kg) produces downregulation of mu receptor mRNA in dorsal root ganglia accompanied by tolerance to the analgesic effects in the hot plate, and the authors suggested that the peripheral nervous system may be important site of opioid tolerance development (Meuser et al., 2003). This form of tolerance is also not surprising when considering that peripheral mu receptors seem to contribute to analgesia produced by systemically administered morphine (Lewanowitsch and Irvine, 2002).

In contrast to studies demonstrating behavioral activity of MRZ 2/576 at doses ≥ 5 mg/kg, Olivar and Laird (1999) reported that this compound inhibited the effects of visceral noxious stimuli (as measured by an increase in blood pressure) with an ED50 of 0.2 mg/kg. These findings support the notion that MRZ 2/576 has peripheral actions, at least in the case of processing of nociceptive signaling.

Another surprising aspect of the present findings is that despite the short half life (of about 15 min) and also the short duration of anticonvulsice activity (Parsons et al., 1997; Hesselink et al., 1999b), MRZ 2/576 was active in the present study when given 30 min before morphine. It is possible that administration of MRZ 2/576 leaves "finger prints", an effect that outlasts the presence of the compound in the body. The "finger print" concept is supported by the data of Belozertseva et al. (2000a), demonstrating that MRZ 2/576 at a dose of 1 mg/kg inhibited spontaneous morphine withdrawal in mice 45–60 min after injection when less than 25% of the injected dose is still present in the body (Parsons et al., 1997; Hesselink et al., 1999b). The "finger print" effect of MRZ 2/576 was also described by Chizh and colleagues in rats in the chronic constriction nerve injury model. In this study MRZ 2/576 (1–10 mg/kg, i.p.) produced an antialloodynic effect more than 24 h after administration of the drug despite the fact that the inhibition of responses to iontophoretically applied NMDA in the spinal cord only lasted about 10–15 min (Chizh et al., 2001). All these and the present findings suggest that even a short lasting blockade of the NMDA receptor/glycineB site may lead to long lasting consequences.

In the present study, both naloxxone hydrochloride and methiodide prevented the development of morphine tolerance with minimal effective doses of 5 and 50 mg/kg, respectively. These doses also inhibited acute morphine antinociception although the former agent was more effective in this regard (Fig. 4). The inhibitory effect of naloxxone methiodide on morphine antinociceptive tolerance may additionally suggest that this phenomenon involves peripheral sites considering that its purity was at least 99% (Sigma–Aldrich communication). The difference in potencies between naloxxone hydrochloride and naloxxone methiodide can be attributed to the difference in the affinity at mu-opioid receptors, for which naloxone methiodide has much lower affinity than naloxxone hydrochloride ca. 50-fold in mu-opioid receptor binding assay and ca. 26 in functional assay (Valentino et al., 1983).

One could also consider the possibility that the NMDA receptor/glycineB site antagonists used in the present study mediate their effects of morphine tolerance in a similar manner to opioid antagonists, but they should then have an inhibitory effect on morphine-induced antinociception per se ("naloxone-like" effect). However, in previous reports, NMDA receptor/glycineB site antagonists, either did not affect, or even potentiated morphine antinociceptive effects in the mice tail-flick test (Lutfy et al., 1999; Belozertseva et al., 2000b), for review see Kozela and Popik (2002), thus making this possibility unlikely. Nevertheless, we investigated this possibility for MRZ 2/596 and this compound did not change morphine’s antinociceptive activity.

Moreover, one cannot exclude the possibility that glycine site NMDA receptor antagonists not penetrating to the brain after acute administration, could show tissue accumulation after chronic administration, leading to concentrations that have central activity. This was investigated in two types of experiments. Treatment with MRZ 2/596 for 7 days did not affect morphine antinociception in the absence of acute MRZ 2/596 and similarly such treatment even at a much higher dose did not influence MES convulsions suggesting insufficient NMDA receptor blockade in the CNS. Thus, the possibility of brain accumulation leading to brain levels sufficient to block NMDA receptors can be rather excluded.

In conclusion, it appears that both the NMDA receptor/glycineB site and opioid receptors in the PNS play a role in the development of antinociceptive tolerance to morphine applied systemically. This further supports previous findings about peripheral (topical) components of morphine tolerance by Kolesnikov and Pasternak group (Kolesnikov et al., 1996; Kolesnikov and Pasternak, 1999a,b).

It can be further hypothesized that the use of NMDA receptor antagonists to inhibit morphine antinociceptive tolerance could be restricted to centrally inactive NMDA receptor antagonists e.g. acting at the NMDA receptor/glycineB sites. Such an approach would allow the avoidance of centrally-mediated side effects known for many NMDA receptor antagonists which penetrate to the brain well.

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