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The antinociceptive effect of combined systemic administration of morphine and the glycine/NMDA receptor antagonist, (+)-HA966 in a rat model of peripheral neuropathy

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1 We evaluated the ability of the functional antagonist at the glycine site of the N-methyl-D-aspartate (NMDA) receptor complex, (+)-(1-Hydroxy-3-aminopyrrolodine-2-one) ((+)-HA966), to modulate the antinociceptive action of systemic morphine in a rat model of neuropathic pain produced by chronic constriction injury to the sciatic nerve. Mechanical (vocalization threshold to hindpaw pressure) and thermal (struggle latency to hindpaw immersion into a water bath) stimuli were used.

2 In the mechanical test, morphine (0.05, 0.1 and 0.3 mg kg^{-1}, i.v.) alone produced dose-dependent effects in both neuropathic and uninjured rats. Likewise, morphine (0.1, 0.3 and 1 mg kg^{-1}, i.v.) dose-dependently increased struggle latencies of the nerve-injured hindpaw in the hot noxious (46°C) test but was ineffective in the non-noxious warm (44°C) and cold (10°C) test.

3 Pretreatment with (+)-HA966 (2.5 mg kg^{-1}, s.c.) dose-dependently enhanced the effect of morphine in the mechanical test with the relative potency being nerve-injured hindpaw > contralateral hindpaw > uninjured rat.

4 Likewise, (+)-HA966 dose-dependently enhanced the effect of morphine against a hot (46°C) stimulus and produced, in combination with morphine, a dose-dependent effect against a warm (44°C) stimulus. In the cold (10°C) test, (+)-HA966 reversed the ineffectiveness of the highest dose of morphine.

5 Naloxone blocked the effect of the combination of (+)-HA966 with morphine in all tests. The drug combination produced no motor deficits in animals using the rotarod test.

6 These findings suggest that combined administration of antagonists, acting at the glycine site of the NMDA receptor complex and morphine may be a promising approach in the treatment of neuropathic and acute pain.

Keywords: Antinociception; neuropathic rat; chronic constriction injury; NMDA receptor antagonist; glycine-site; (+)-HA966; morphine; vocalization threshold; struggle latency

Introduction

Damage to the peripheral nervous system often leads to abnormal pain states referred to as neuropathic pain. This pain syndrome consists of some specific somatosensory disorders. The most prominent symptoms include (1) allodynia (innocuous stimulation evokes abnormally intense, prolonged pain sensations), and (2) hyperalgesia (noxious stimulation evokes abnormally intense and prolonged pain sensations). These sensations can be provoked by both mechanical and thermal (heat or cold) stimulation (Payne, 1986).

Several lines of evidence indicate that the N-methyl-D-aspartate (NMDA) receptor is involved in the induction and maintenance of hypersensitivity states associated with chronic pain, including neuropathic pain. NMDA receptor antagonists block pain transmission in dorsal horn spinal neurons (Dickenson & Sullivan, 1987; Seltzer *et al*., 1991) and reduce pain-related behaviour in neuropathic animal models (Davar *et al*., 1991; Mao *et al*., 1993). Despite their efficacy in clinical trials (Backonja *et al*., 1994; Eide *et al*., 1994; Pud *et al*., 1998) human use of NMDA receptor antagonists has been limited by potentially serious neurotoxic side effects.

In contrast to NMDA receptor antagonists, the effect of morphine in neuropathic pain states has been a matter of considerable controversy. While often classified as opioid resistant (Arner & Meyerson, 1988), it is now generally accepted that neuropathic pain exhibits a reduced sensitivity to systemic opiates (Portenoy & Foley, 1986; Jadad *et al*., 1992). We have previously shown, that in the chronic constriction injury (CCI) rat model of neuropathy (Bennett & Xie, 1988; Attal *et al*., 1990), systemic morphine produces dose-dependent antinociceptive effects against a mechanical (Attal *et al*., 1991; Kayser *et al*., 1995b; Catheline *et al*., 1996; Idänpään-Heikkilä *et al*., 1997) and a noxious hot (46°C) stimulus (Lee *et al*., 1994; Idänpään-Heikkilä *et al*., 1997). In contrast, morphine was ineffective against a non-noxious cold (10°C) (Lee *et al*., 1994; Idänpään-Heikkilä *et al*., 1997) and a non-noxious warm (44°C) stimulus (Lee *et al*., 1994).

A major contributor to a decreased responsiveness of morphine in neuropathic pain states could be the activation of the NMDA receptor (Chapman *et al*., 1994; Dickenson, 1997). It has been shown that NMDA receptor antagonism reverses the ineffectiveness of morphine in depressing dorsal horn neuronal activity (Chapman & Dickenson, 1992). In addition, the sensitivity of thermal hyperalgesia (Yamamoto & Yaksh, 1992; Ossipov *et al*., 1995) and mechanical allodynia (Nichols *et al*., 1997) to intrathecal morphine can be restored by concomitant intrathecal administration of a NMDA receptor antagonist in different models of neuropathic pain.

Activation of the NMDA receptor complex requires occupation of recognition sites by both glutamate and glycine

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(see Kemp & Leeson, 1993) and antagonism at the glycine recognition site reduces spinal nociception in the rat (Dickenson & Aydar, 1991). The functional antagonist at the glycine site of the NMDA receptor complex, (+)-HA966, has been shown to cross the blood-brain barrier and to be associated with less motor side effects than original NMDA receptor antagonists (see Kemp & Leeson, 1993; Millan & Seguin, 1993). In a recent study, coadministration of systemic morphine with (+)-HA966 profoundly reduced inflammation-evoked spinal c-*fos* expression (Honore *et al*., 1996).

In the present experiments we studied the ability of (+)-HA966 to modulate the antinociceptive effect of systemic morphine against mechanical and noxious hot stimuli in the CCI rat model of neuropathic pain. We also wanted to test if the glycine/NMDA receptor antagonist is able to reveal an effect of morphine against warm and cold stimuli.

Methods

The Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP) Ethical Guidelines (1983) were adhered to in these studies.

Animals

Male Sprague-Dawley rats (Charles River, France, strain designation Crl:CD(SD)BR), $n=370$, weighing 175–200 g on arrival were used. The rats were housed five to a cage on a 12 h light/12 h dark cycle. The ambient temperature was kept at 22°C, and the rats had free access to standard laboratory food and tap water. The animals were allowed to habituate to the housing facilities for at least 1 week before the experiments were begun.

Surgical procedure

The unilateral peripheral mononeuropathy was produced on the right hindpaw according to the method described by Bennett & Xie (1988) and Attal *et al*. (1990). In brief, the animals were anaesthetized with sodium pentobarbitone (Nembutal, 50 mg kg^{-1}, i.p.). The common sciatic nerve was exposed by blunt dissection at the level of the mid-thigh and four ligatures (5-0 chromic catgut, about 1 mm spacing) were placed around the nerve taking care not to interrupt the epineural circulation. To minimize the discomfort and possible painful mechanical stimulation, the rats were housed in large cages with saw dust bedding after the surgery. The neuropathic rats were able to eat and drink unaided.

Mechanical and thermal testing

Neuropathic rats were used 2 weeks after surgery. At this time, as described previously, the abnormal pain behaviour is at a stable maximum (Bennett & Xie, 1988; Attal *et al*., 1990). All experiments were carried out in a quiet room between 08 00 h and 15 00 h. The animals were randomly assigned in groups of five (mechanical test) or ten (thermal tests) for a given series of tests and were not acclimatized to the test situations beforehand. The experiments were performed by two different experimenters unaware of the drug combinations used. Each animal received drugs only once and was used in only one experiment. Testing sessions lasted for 2–4 h and at the end of the experiment rats were killed.

In mechanical tests, the antinociceptive action was determined by measuring the vocalization threshold elicited by pressure on the right hindpaw in uninjured rats, and on both the nerve-injured and the contralateral hindpaw in neuropathic rats, using the Ugo Basile (Comerio, Italy) analgesymeter. This instrument generates a linearly increasing mechanical force applied by a dome shaped plastic tip (diameter = 1 mm) on the dorsal surface of the paw. The tip was positioned between the third and fourth metatarsus (into the sciatic nerve territory) and force was applied until the rat squeaked. This centrally integrated response is especially sensitive to analgesic compounds, particularly in this model of neuropathy (Attal *et al*., 1991; Kayser *et al*., 1995b; Catheline *et al*., 1996; Idänpään-Heikkilä *et al*., 1997). For each rat, a control threshold (mean of two consecutive stable thresholds expressed in g) was determined before injecting the drugs. Over the first 20 min following drug administration, the vocalization thresholds were measured every 5 min and thereafter every 10 min, until they had returned to the level of the control values.

Thermal nociception was tested by measuring the struggle latency elicited by immersion of the nerve-injured hindpaw into a 10°C (non-noxious range, Guilbaud *et al*., 1990), 44°C (at the noxious threshold, Guilbaud *et al*., 1990) or 46°C (clearly in the noxious range) water bath (Ministat MHUB 11, Bioblock Scientific, France) as extensively described elsewhere (Attal *et al*., 1990; Lee *et al*., 1994; Idänpään-Heikkilä *et al*., 1997). For each rat, a control latency (mean of two consecutive trials 20 min apart, expressed in s) was determined before injecting the drugs. After drug administration, struggle latencies were measured every 20 min until they had returned to baseline. The long interval (20 min) between successive measurements was necessary, as in this model, abnormal reactions lasting for more than 15 min following a thermal stimulus have been reported (Attal *et al*., 1990; Guilbaud *et al*., 1990).

Motor coordination testing

Locomotor function was tested using the Ugo Basile accelerating rotarod (model 7750) for rats. This apparatus consists of a base platform and a rotating rod of 7 cm diameter with a non-skid surface. The rod, 50 cm in length, is divided into four equal sections by five disks and four rats can be tested simultaneously. Under each drum section, 26 cm below the rod on the base platform, a V-shaped counter-trip plate is positioned. The animals were acclimatized to the revolving drum and habituated to handling in order to avoid stress during testing. The rod was set to accelerate from 4 to 40 r.p.m. in a period of 5 min. The integrity of motor coordination was assessed as the performance time on the rod measured from the start of acceleration until the animal fell from the drum onto the counter-trip plate. The rats were acclimatized to acceleration by three training runs. The mean of the fourth and fifth training run served as control performance time (expressed in s). After drug administration, performance time was measured every 20 min for a total of 120 min.

Drugs and doses

The following drugs were used: morphine hydrochloride (Meram, Paris, France), naloxone hydrochloride (Narcan®, Du Pont Pharma, Paris, France), (+)-HA966 ((+)-(1-Hydroxy-3-aminopyrrolodine-2-one)), (Tocris Cookson, Bristol, England) and saline (0.9% NaCl w v^{-1}). Morphine and naloxone were diluted in saline and administered i.v. in a volume of 1 ml kg^{-1} into the lateral tail vein. The doses of morphine used in mechanical (0.05, 0.1 and 0.3 mg kg^{-1}) and

thermal (0.1, 0.3 and 1 mg kg^{-1}) tests were based on our previous experiments, showing a higher sensitivity of mechanical stimuli to morphine (Attal *et al*., 1991; Idänpään-Heikkilä *et al*., 1997). Naloxone (0.1 mg kg^{-1}) was coinjected with morphine at a dose that has been shown to prevent the effect of 1 mg kg^{-1} of morphine (Idänpään-Heikkilä *et al*., 1997). (+)-HA966 was dissolved in saline and administered s.c. in a volume of 1 ml kg^{-1} 20 min before morphine. The dose, 2.5 mg kg^{-1} of (+)-HA966 , was chosen since given alone it produced no antinociceptive effect in a study on inflammatory pain in the rat (Chapman *et al*., 1995). In each group, the control rats received the same volume of i.v. or s.c. saline.

Statistics

Data are expressed as means ± s.e.mean. The areas under the curves (AUC) were calculated using the trapezoidal rule. Values in g (vocalization thresholds) or s (struggle latencies) were used for the statistical analyses. Statistical significance of the data was analysed by one-way analysis of variance (ANOVA). The observed significances were then confirmed with Tukey's test. Simple regressions (linear model) were performed to establish dose-dependent effects. All procedures were carried out using a computer program (Statgraphics Plus, Manugistics, Rockville, Maryland, U.S.A.). The observed differences were regarded as being significant when the P values were lower than 0.05.

Table 1 Maximal mean vocalization thresholds from the nerve-injured and contralateral hindpaws as well as in uninjured rats in the paw pressure test before and after injection of saline + morphine or (+)-HA966 (2.5 mg kg^{-1} s.c.) + morphine

	Nerve-injured hindpaw		*Contralateral hindpaw*			*Uninjured rat*		
Treatment	*Before injury (g)*	*After injury (g)*	*Before injury (g)*	*After injury (g)*	n	*Before injury (g)*	*After injury (g)*	n
Saline + saline	189 ± 29	195 ± 29	254 ± 23	251 ± 27	7	N.D.	N.D.	
(+)-HA966 + saline	159 ± 12	171 ± 4	228 ± 16	246 ± 12	5	239 ± 8	249 ± 6	5
Saline + morphine 0.05	158 ± 10	180 ± 15	243 ± 18	253 ± 24	6	230 ± 8	237 ± 7	5
(+)-HA966 + morphine 0.05	143 ± 8	215 ± 23#	237 ± 20	306 ± 27	10	230 ± 18	240 ± 15	5
Saline + morphine 0.1	167 ± 11	234 ± 16*	260 ± 13	296 ± 17	8	231 ± 8	258 ± 11	5
(+)-HA966 + morphine 0.1	205 ± 12	460 ± 38#	288 ± 7	402 ± 55	9	205 ± 6	261 ± 12#	8
Saline + morphine 0.3	168 ± 7	243 ± 13#	248 ± 9	313 ± 16*	9	233 ± 7	289 ± 11#	7
(+)-HA966 + morphine 0.3	185 ± 10	582 ± 49#	251 ± 6	443 ± 53#	9	193 ± 9	304 ± 5#	8
(+)-HA966 + morphine 0.3 + Nx (0.1 mg kg^{-1})	128 ± 6	138 ± 5	208 ± 16	213 ± 19	6	229 ± 7	223 ± 7	6

Results are expressed as means ± s.e.mean. The after injection (inj) values are the peak effects. *P < 0.05, #P < 0.01 vs before injection (Tukey's test). N.D.-not determined. Morphine (mg kg^{-1} i.v.) was injected 20 min after (+)-HA966. Naloxone (Nx) was coinjected with morphine.

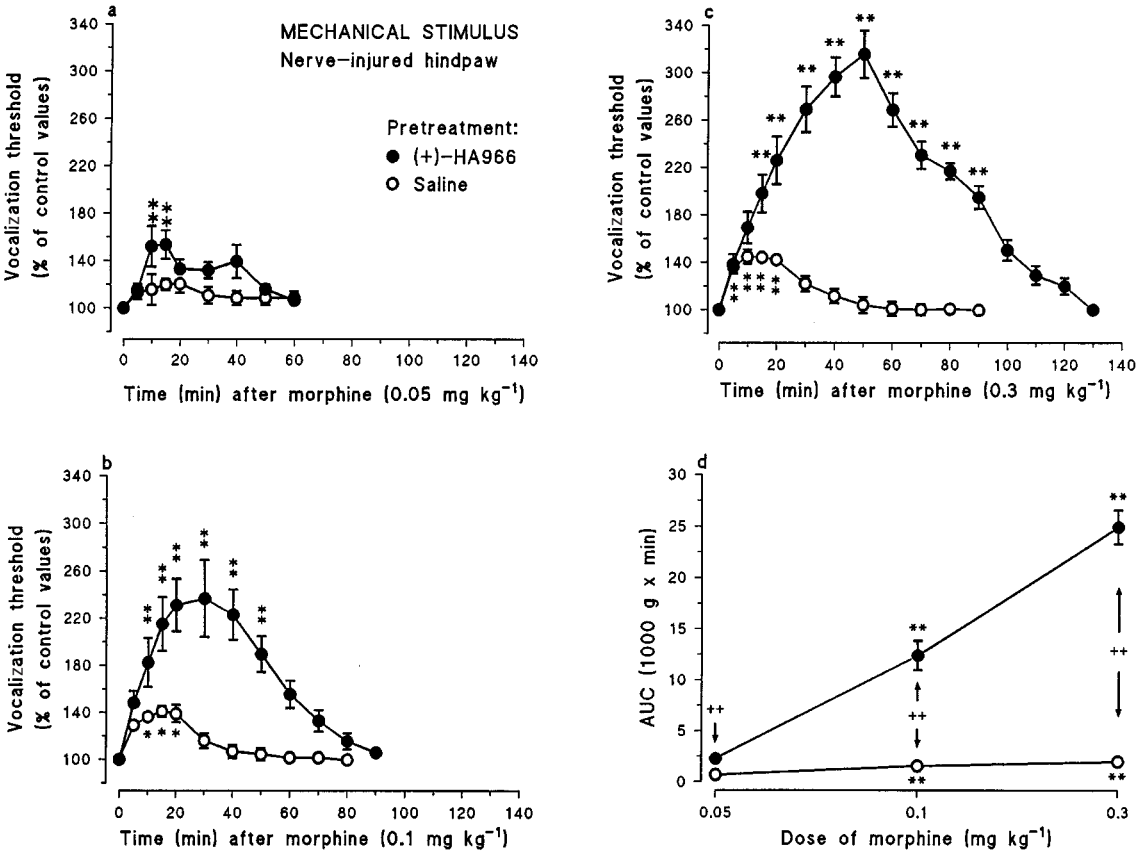

Figure 1 Effect of i.v. morphine on the vocalization threshold to pressure on the nerve-injured hindpaw in neuropathic rats after pretreatment with (+)-HA966 2.5 mg kg^{-1} (s.c.) or saline s.c. (a–c). (a) morphine 0.05 mg kg^{-1} (b) morphine 0.1 mg kg^{-1} (c) morphine 0.3 mg kg^{-1} (d) AUCs (1000 g × min) of the respective time-curves. Data (means ± s.e.mean of n = 5–10) in a–c are given as percentages of the corresponding control values. Statistics were calculated with vocalization thresholds expressed in g: *P < 0.05, **P < 0.01 vs control; ^{+}P < 0.05, ^{++}P < 0.01 vs pretreatment with saline, Tukey's test.

Results

In agreement with previous studies, (see Idänpään-Heikkilä *et al*., 1997 and references therein) the vocalization threshold of the nerve-injured hindpaw was decreased 2 weeks after the CCI-producing surgery (169±4 g vs the preconstriction value 240±8 g, $P<0.001$, $n=69$). This decreased threshold was considered to reflect mechanical allodynia (Merskey, 1986). The threshold of the contralateral hindpaw was not modified (250±5 g vs the preconstriction value 245±6 g, $n=69$). In uninjured rats the vocalization threshold was 222±4 g ($n=49$).

As also reported earlier (Lee *et al*., 1994; Idänpään-Heikkilä *et al*., 1997) the struggle latencies at 46°C (5.6±0.3 s vs the preconstriction value 8.1±0.2 s, $P<0.001$, $n=77$), 44°C (7.9±0.2 s vs the preconstriction value 14.6±0.1 s, $P<0.001$, $n=60$) and 10°C (6.1±0.2 s vs the preconstriction value 14.9±0.9 s, $P<0.001$, $n=53$) were decreased. We considered the decreases in struggle latencies against warm (44°C) and cold (10°C) stimuli to reflect thermal allodynia (Merskey, 1986).

Effect of (+)-HA966 and morphine against a mechanical stimulus

In the nerve-injured hindpaw, saline, (+)-HA966 alone or morphine (0.05 mg kg^{-1}) alone (Table 1, Figure 1a) had no effect on the vocalization threshold. The effect of the combination of (+)-HA966 and morphine (0.05 mg kg^{-1}) peaked (154±12%) at and lasted for 15 min ($P<0.001$, Table 1, Figure 1a). Both morphine (0.1 mg kg^{-1}) alone and the combination of (+)-HA966 and morphine (0.1 mg kg^{-1}) resulted in a significant elevation of the vocalization threshold ($P<0.001$ for both, Figure 1b). The effect of morphine (0.1 mg kg^{-1}) alone peaked (144±7%) at 15 min and lasted for 20 min. The effect of the combination peaked (237±33%) at 30 min and lasted for 50 min (Table 1, Figure 1b). The vocalization threshold increased significantly after administration of both morphine (0.3 mg kg^{-1}) alone and (+)-HA966 in combination with this dose of morphine ($P<0.01$ and $P<0.001$ respectively, Figure 1c). The effect of morphine (0.3 mg kg^{-1}) alone peaked (153±10%) at 10 min and lasted for 20 min whereas the effect of the combination peaked (315±21%) at 50 min and lasted for 90 min (Table 1, Figure 1c).

In the contralateral hindpaw, saline, (+)-HA966 alone or morphine alone at 0.05 mg kg^{-1} and 0.1 mg kg^{-1} had no effect (Table 1, Figure 2a,b). The combination of (+)-HA966 with morphine (0.05 mg kg^{-1} or 0.1 mg kg^{-1}) resulted in a moderate overall antinociceptive effect ($P<0.05$ and $P<0.01$ respectively, Table 1, Figure 2a,b). The vocalization threshold increased significantly after administration of both morphine (0.3 mg kg^{-1}) alone and (+)-HA966 in combination with this dose of morphine ($P<0.001$ for both, Figure 2c). The effect of morphine (0.3 mg kg^{-1}) alone peaked (129±5%) at and lasted for 15 min, whereas the effect of the combination peaked (176±19%) at 50 min and lasted for 60 min (Table 1, Figure 2c).

In uninjured rats, no antinociception was produced by either (+)-HA966 alone, lower doses of morphine (0.05 and

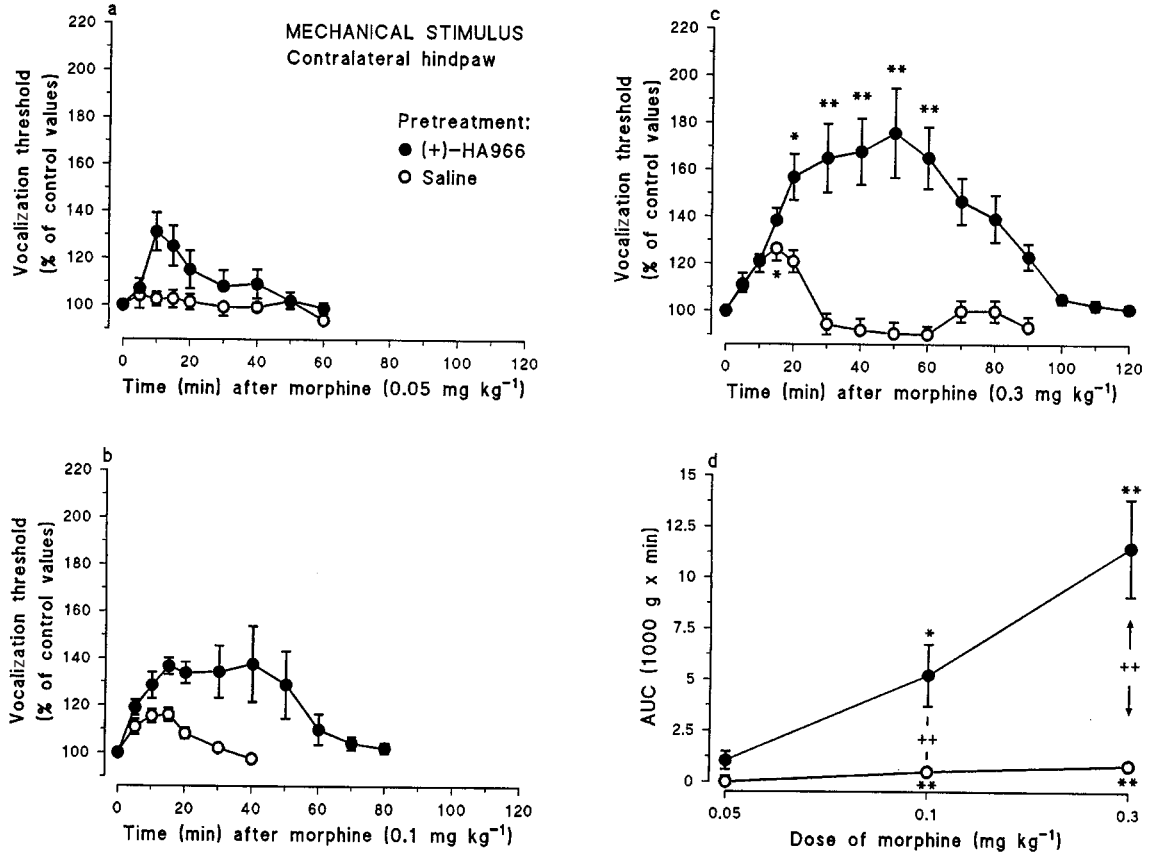

Figure 2 Effect of i.v. morphine on the vocalization threshold to pressure on the contralateral hindpaw in neuropathic rats after pretreatment with (+)-HA966 2.5 mg kg^{-1} (s.c.) or saline s.c. (a–c). (a) morphine 0.05 mg kg^{-1}, (b) morphine 0.1 mg kg^{-1}, (c) morphine 0.3 mg kg^{-1}, (d) AUCs (1000 g×min) of the respective time-curves. Data (means±s.e.mean of $n=5-10$) in a–c are given as percentages of the corresponding control values. Statistics were calculated with vocalization thresholds expressed in g: $^{*}P<0.05$, $^{**}P<0.01$ vs control; $^{+}P<0.05$, $^{++}P<0.01$ vs pretreatment with saline, Tukey's test.

0.1 mg kg^{-1}) alone or the combination of (+)-HA966 with morphine (0.05 mg kg^{-1}) (Table 1, Figure 3a,b). By contrast, the combination of (+)-HA966 with morphine at 0.1 mg kg^{-1} resulted in a significant antinociceptive effect ($P<0.001$) that peaked (127±6%) at 15 min and lasted for 20 min (Table 1, Figure 3b). The vocalization threshold also increased significantly after administration of both morphine (0.3 mg kg^{-1}) alone and (+)-HA966 in combination with this dose of morphine ($P<0.001$ for both, Figure 3c). The effect of morphine (0.3 mg kg^{-1}) alone peaked (123±3%) at 15 min and lasted for 20 min, whereas the effect of the combination peaked (160±8%) at 20 min and lasted for 30 min (Table 1, Figure 3c).

Significant dose-effect relationships were observed for morphine alone in both the nerve-injured ($r=0.50$, $P<0.5$, Figure 1d) and the contralateral ($r=0.57$, $P<0.01$, Figure 2d) hindpaw as well as in uninjured rats ($r=0.76$, $P<0.001$, Figure 3d). Pretreatment with (+)-HA966 enhanced the overall effect of morphine ($P<0.001$, $P<0.001$ and $P<0.01$ for the nerve-injured hindpaw, the contralateral hindpaw and uninjured rats, respectively) and produced a significant dose-effect relationship in both the nerve-injured ($r=0.92$, $P<0.001$, Figure 1d) and the contralateral ($r=0.69$, $P<0.001$, Figure 2d) hindpaw as well as in uninjured rats ($r=0.83$, $P<0.001$, Figure 3d).

Effect of (+)-HA966 and morphine against a hot (46°C) stimulus

Saline, (+)-HA966 alone or morphine (0.1 mg kg^{-1}) alone had no effect on the struggle latency at 46°C (Table 2, Figure 4a). The combination of (+)-HA966 with morphine (0.1 mg kg^{-1}) produced an effect that peaked (165±15%) at and lasted for 60 min ($P<0.01$, Table 2, Figure 4a). Morphine alone at 0.3 mg kg^{-1} caused a significant effect peaking (134±5%) at and lasting for 20 min ($P<0.001$, Table 2, Figure 4b). Similarly, the combination of (+)-HA966 and morphine (0.3 mg kg^{-1}) produced an effect that peaked (181±22%) at and lasted for 100 min ($P<0.001$, Table 2, Figure 4b). Both morphine (1 mg kg^{-1}) alone and the combination of (+)-HA966 with morphine (1 mg kg^{-1}) increased the struggle latency significantly ($P<0.01$ and $P<0.001$ respectively, Figure 4c). The effect of morphine (1 mg kg^{-1}) alone peaked (184±13%) at and lasted for 40 min. The effect of the combination peaked (243±39%) at 60 min and lasted for 80 min (Table 2, Figure 4c).

As shown in Figure 4d, dose-effect relationships were observed for both morphine ($r=0.74$, $P<0.001$) and the combination of (+)-HA966 with morphine ($r=0.55$, $P<0.001$). Pretreatment with (+)-HA966 enhanced the overall effect of morphine ($P<0.001$, Figure 4d).

Effect of (+)-HA966 and morphine against a warm (44°C) stimulus

Saline, (+)-HA966 alone or morphine alone had no effect against a warm (44°C) stimulus (Table 2, Figure 5). In the (+)-HA966-pretreated groups, the lowest dose of morphine (0.1 mg kg^{-1}) was unable to modify the struggle latency (Table 2, Figure 5a). In contrast, pretreatment with the

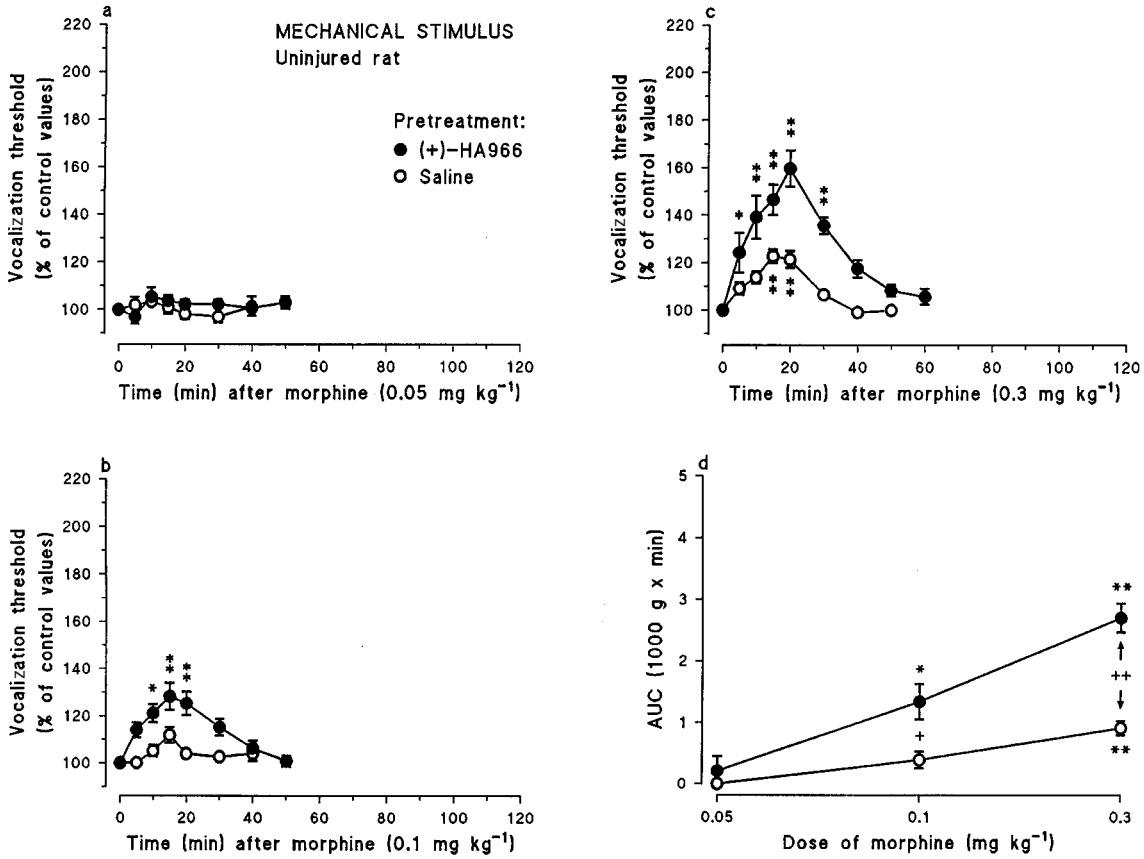

Figure 3 Effect of i.v. morphine on the vocalization threshold to hindpaw pressure in uninjured rats after pretreatment with (+)-HA966 2.5 mg kg^{-1} (s.c.) or saline s.c. (a–c). (a) morphine 0.05 mg kg^{-1}, (b) morphine 0.1 mg kg^{-1}, (c) morphine 0.3 mg kg^{-1}, (d) AUCs (1000 g × min) of the respective time-curves. Data (means±s.e.mean of $n=5-8$) in a–c are given as percentages of the corresponding control values. Statistics were calculated with vocalization thresholds expressed in g: $^{*}P<0.05$, $^{**}P<0.01$ vs control; $^{+}P<0.05$, $^{++}P<0.01$ vs pretreatment with saline, Tukey's test.

glycine/NMDA receptor antagonist reversed the ineffectiveness of the two higher doses (0.3 and 1 mg kg^{-1}) of morphine ($P<0.05$ and $P<0.01$ respectively, Figure 5b,c). The effect of the combination of (+)-HA966 and morphine (0.3 mg kg^{-1}) peaked (150±8%) at and lasted for 40 min. The effect of the combination of (+)-HA966 with morphine (1 mg kg^{-1}) peaked (188±16%) at 40 min and lasted for 100 min (Table 2, Figure 5b,c). The combination of (+)-HA966 with morphine produced a significant dose-effect relationship ($r=0.63$, $P<0.001$, Figure 5d).

Effect of (+)-HA966 and morphine against a cold (10°C) stimulus

Neither saline, (+)-HA966 alone or any of the three doses (0.1, 0.3 and 1 mg kg^{-1}) of morphine alone was able to modify struggle latencies in the cold test (Table 2). The two lower doses (0.1 and 0.3 mg kg^{-1}) of morphine were still ineffective after pretreatment with (+)-HA966 (Table 2). By contrast, an increase in the struggle latency was observed in the (+)-HA966-pretreated group after morphine at 1 mg kg^{-1}

Table 2 Maximal mean struggle latencies elicited by immersion of the nerve-injured hindpaw into a hot (46°C), warm (44°C) or cold (10°C) water bath before and after injection of saline+morphine or (+)-HA966 (2.5 mg kg^{-1} s.c.)+morphine

	46°C			*44°C*			*10°C*		
Treatment	*Before injury (s)*	*After injury (s)*	n	*Before injury (s)*	*After injury (s)*	n	*Before injury (s)*	*After injury (s)*	n
Saline+saline	4.3±0.3	4.5±0.4	6	8.6±1.1	8.6±1.0	5	4.2±0.3	4.0±0.5	5
(+)-HA966+saline	4.1±0.1	4.6±0.2	6	8.7±0.7	10.1±0.9	5	8.8±0.6	9.8±0.7	5
Saline+morphine 0.1	6.0±0.7	6.2±0.9	12	7.8±0.7	8.4±1.8	5	4.6±0.2	4.6±0.5	5
(+)-HA966+morphine 0.1	7.5±0.6	11.8±0.9*	12	7.8±0.4	8.8±1.2	6	5.9±0.6	6.1±0.5	5
Saline+morphine 0.3	5.4±0.4	7.3±0.5*	9	8.2±0.5	9.6±0.2	5	5.7±0.4	6.2±0.3	5
(+)-HA966+morphine 0.3	6.2±0.4	11.2±1.4*	9	8.0±0.5	12.0±0.9*	10	6.2±0.5	7.6±1.3	8
Saline+morphine 1	4.3±0.3	8.1±1.0*	9	7.7±0.7	10.3±1.2	9	5.9±0.8	6.6±0.6	6
(+)-HA966+morphine 1	5.2±0.4	11.1±1.2#	9	7.2±0.4	13.2±0.8#	9	6.1±0.5	13.8±0.5#	8
(+)-HA966+morphine 1 +Nx (0.1 mg kg^{-1})	5.0±0.6	5.2±1.5	5	8.1±0.8	9.5±1.7	6	6.9±0.7	9.9±0.5	6

Results are expressed as means±s.e.mean. The after injection (inj) values are the peak effects. *$P<0.05$, #$P<0.01$ vs before injection (Tukey's test). Morphine (mg kg^{-1} i.v.) was injected 20 min after (+)-HA966. Naloxone (Nx) was coinjected with morphine.

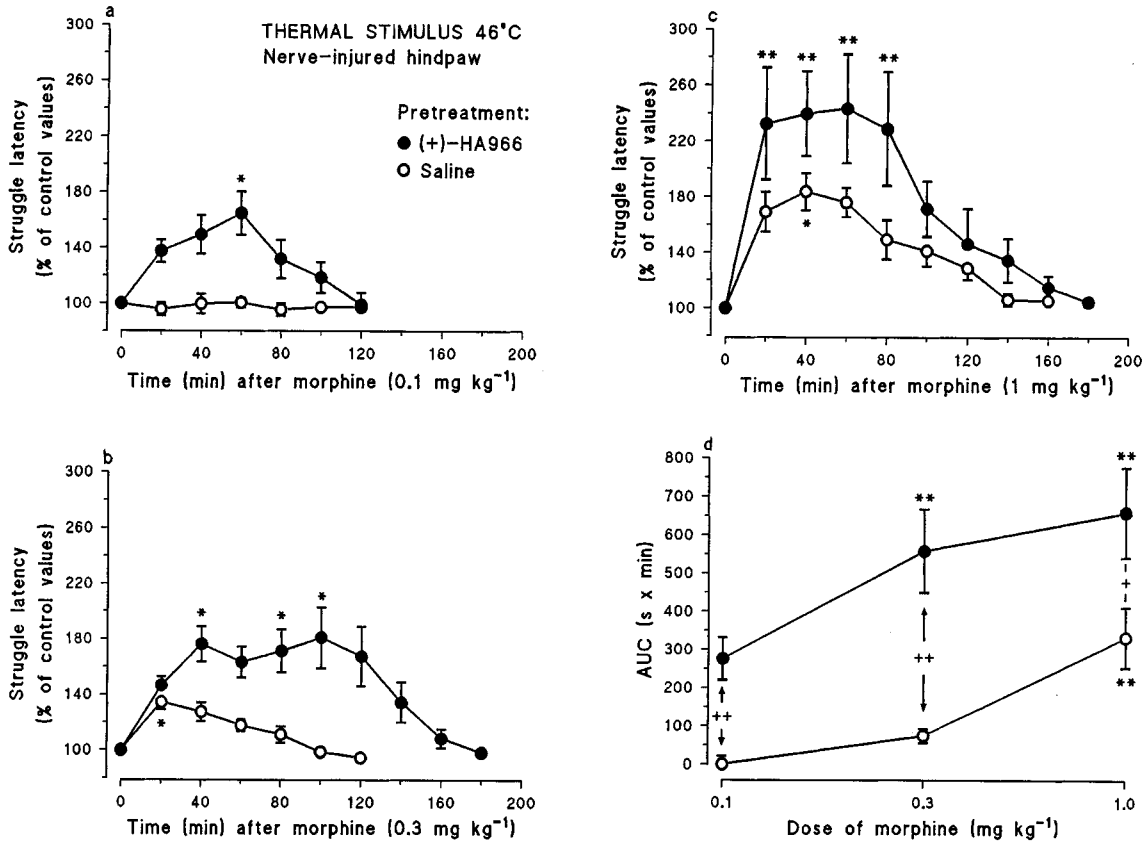

Figure 4 Effect of i.v. morphine on the struggle latency to immersion of the nerve-injured hindpaw of neuropathic rats into a hot (46°C) water bath after pretreatment with (+)-HA966 2.5 mg kg^{-1} (s.c.) or saline s.c. (a–c). (a) morphine 0.1 mg kg^{-1}, (b) morphine 0.3 mg kg^{-1}, (c) morphine 1 mg kg^{-1}, (d) AUCs (s×min) of the respective time-curves. Data (means±s.e.mean of $n=5-12$) in a–c are given as percentages of the corresponding control values. Statistics were calculated with struggle latencies expressed in s: *$P<0.05$, **$P<0.01$ vs control; $^{+}P<0.05$, $^{++}P<0.01$ vs pretreatment with saline, Tukey's test.

($P<0.001$, Figure 6). The effect peaked (236±21%) at 80 min and lasted for 120 min (Table 2, Figure 6).

Reversal of the antinociceptive effect by naloxone

In the mechanical test (Table 1), naloxone (0.1 mg kg^{-1}) blocked the effect of the combination of (+)-HA966 and morphine (0.3 mg kg^{-1}). Similarly, in thermal tests (Table 2) the effect of morphine (1 mg kg^{-1}) combined with (+)-HA966 was abolished by naloxone (0.1 mg kg^{-1}).

Effect of the combination of (+)-HA966 and morphine on motor coordination

The locomotor function of the neuropathic rats was evaluated using the rotarod (accelerating) test. We observed no change in rotarod performance time after injecting the combination of (+)-HA966 and morphine (1 mg kg^{-1}) (Table 3).

Discussion

(+)-HA966 dose-dependently enhances the effect of morphine against mechanical and hot (46°C) stimuli

In agreement with numerous previous studies (see Idänpään-Heikkilä *et al*., 1997 and references therein), morphine alone dose-dependently increased the vocalization threshold in the mechanical test. The effect of morphine appeared more potent on the nerve-injured hindpaw than on the contralateral hindpaw, and the effect on the contralateral hindpaw was comparable to that observed in uninjured rats as shown earlier (Attal *et al*., 1991). Similarly, in the noxious thermal range

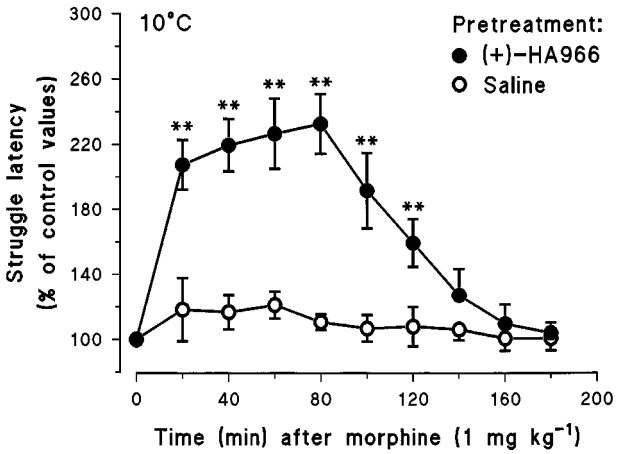

Figure 6 Effect of i.v. morphine (1 mg kg^{-1}) on the struggle latency to immersion of the nerve-injured hindpaw of neuropathic rats into a cold (10°C) water bath after pretreatment with (+)-HA966 2.5 mg kg^{-1} (s.c.) or saline s.c. Data (means±s.e.mean of $n=5-8$) are given as percentages of the corresponding control values. Statistics were calculated with struggle latencies expressed in s: $^{*}P<0.05$, $^{**}P<0.01$ vs control, Tukey's test.

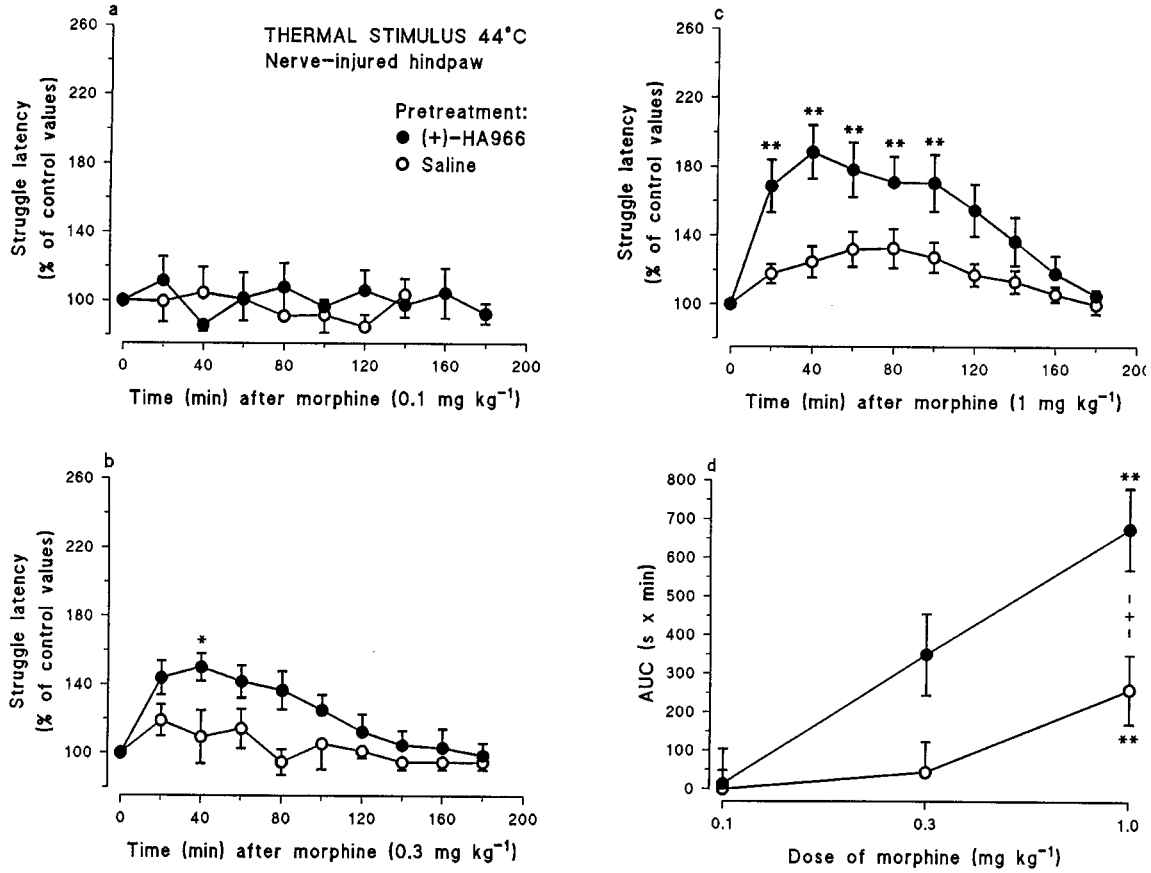

Figure 5 Effect of i.v. morphine on the struggle latency to immersion of the nerve-injured hindpaw of neuropathic rats into a warm (44°C) water bath after pretreatment with (+)-HA966 2.5 mg kg^{-1} (s.c.) or saline s.c. (a–c). (a) morphine 0.1 mg kg^{-1}, (b) morphine 0.3 mg kg^{-1}, (c) morphine 1 mg kg^{-1}, (d) AUCs (s×min) of the respective time-curves. Data (means±s.e.mean of $n=5-10$) in a–c are given as percentages of the corresponding control values. Statistics were calculated with struggle latencies expressed in s: $^{*}P<0.05$, $^{**}P<0.01$ vs control; $^{+}P<0.05$, $^{++}P<0.01$ vs pretreatment with saline, Tukey's test.

Table 3 Rotarod performance time before and every 20 min after injection of (+)-HA966 (2.5 mg kg^{-1} s.c.)+morphine (1 mg kg^{-1} i.v.) in neuropathic rats

Treatment	*Before injection* (s)	*Post-injection time* 20 min (s)	40 min (s)	60 min (s)	80 min (s)	100 min (s)	120 min (s)	n
Saline+saline	67±5	63±5	55±3	80±8	69±7	68±9	65±7	8
(+)-HA966+morphine	69±7	60±8	72±6	97±7	72±9	75±6	75±13	8

Results are expressed as means±s.e.mean. Morphine was injected 20 min after (+)-HA966.

(46°C), morphine produced a dose-dependent antinociceptive effect.

In this study, we demonstrate that a profound antinociceptive effect is obtained by concomitant administration of the glycine/NMDA receptor antagonist, (+)-HA966 and morphine. This result fits in well with results from previous studies in different models of neuropathic pain using either systemic (Advokat & Rhein, 1993; Kauppila *et al*., 1998) or intrathecal (Yamamoto & Yaksh, 1992; Ossipov *et al*., 1995; Nichols *et al*., 1997) routes of administration. In these studies, however, the NMDA receptor antagonist failed to modulate the effect of morphine in the contralateral paw (Yamamoto & Yaksh, 1992) or in sham-operated rats (Ossipov *et al*., 1995). This contrasts with our results, in which the effect of the combination of (+)-HA966 and morphine remained significant and dose-dependent in the contralateral hindpaw as well as in uninjured rats with the relative potency being nerve-injured hindpaw > contralateral hindpaw > uninjured rat. Comparisons between studies are, however, difficult because of differences in drugs used, in models of neuropathy, in nociceptive tests and in routes of administration. Our data are supported by studies in uninjured rats showing a potentiation of the antinociceptive effect of morphine by different NMDA receptor antagonists (Kest *et al*., 1992; Grass *et al*., 1996). The drugs in these previous studies were also injected systemically, which emphasizes the importance of the route of administration when comparing analgesic properties of drugs.

The potent antinociceptive effect of the drug combination in the nerve-injured hindpaw is consistent with the current hypothesis of the physiological role of the NMDA receptor and the μ-opioid receptor system in neuropathic pain (Dickenson, 1997). The development of neuropathic pain involves an increase in the spontaneous activity of injured nerves, which may underlie the increase in excitability of neurones in the spinal cord (Woolf, 1983). These phenomena are behaviourally manifested as allodynia and hyperalgesia. There is evidence that these events are mediated, in part, by excitatory amino acids (EAA) acting on the NMDA receptor in the spinal dorsal horn (Dickenson & Sullivan, 1990; Woolf & Thompson, 1991). Spinal morphine mainly mediates its effect through μ-opioid receptors on primary afferent terminals. It has been shown that activation of presynaptic μ-opioid receptors reduces dorsal root stimulation-evoked outflow of EAA (Kangra & Randic, 1991). Consequently, in the present study the supra-additive effect of the combination of morphine and (+)-HA966 on the nerve-injured hindpaw could be due to a functional potentiation between the separate effects of the two receptor systems with morphine reducing presynaptic neurotransmitter release, thus lowering the amount of EAA available for the NMDA receptor and (+)-HA966 acting post-synaptically further reducing the NMDA receptor mediated events leading to sensitization of the postsynaptic cell. The antinociception so caused is mainly mediated *via* opioid receptors since it may be blocked by naloxone.

The mechanisms proposed above can hardly account for the dose-dependent effect of the combination of (+)-HA966 with morphine observed in the contralateral hindpaw of neuropathic rats or in uninjured rats, since under these conditions the NMDA receptor is presumably in a non-activated state. However, in addition to its presynaptic action, morphine exerts, a direct μ-opioid receptor mediated inhibition of the postsynaptic cell, which raises possibilities for a direct mechanism of interaction between the two receptor systems (see Wiesenfeld-Hallin, 1998). It has been shown, that μ-opioid receptor agonism enhances NMDA-induced membrane currents in isolated dorsal horn neurons (Chen & Huang, 1991; Rusin & Randic, 1991). Based on this and other observations it has been suggested that a single administration of morphine is able to activate the NMDA-receptor, leading to acute tolerance to the antinociceptive effect of morphine (see Wiesenfeld-Hallin, 1998). If such mechanisms are operating in our study, acute morphine injection would induce a hypersensitivity state in the post-synaptic cell, that counteracts the inhibitory opiate effect, resulting in a reduced antinociceptive effect of morphine. Concomitant antagonism at the NMDA receptor may therefore enhance the effect of morphine also in acute pain.

Finally, both NMDA and opioid receptors are abundantly represented in extra-spinal nociceptive pathways, and a supraspinal as well as a peripheral component may contribute to the pronounced antinociceptive effect observed in this study. Indeed, previous studies by our group suggest, that peripheral opioid receptor mechanisms participate in the antinociceptive effect of i.v. morphine in the present model of neuropathic pain (Kayser *et al*., 1995b; Catheline *et al*., 1996). In addition, there is evidence of the presence of NMDA receptors on axons in the glabrous skin of the rat hindpaw (Carlton *et al*., 1995). Furthermore, it has recently been reported that intraplantar injection of NMDA in the rat results in mechanical allodynia and hyperalgesia, which can be attenuated by a local injection of a non-competitive NMDA receptor antagonist (Zhou *et al*., 1996). An eventual peripheral contribution to the antinociceptive effect of the combination of morphine and NMDA receptor antagonists will have to be elucidated in future studies.

(+)-HA966 reverses the ineffectiveness of morphine against warm (44°C) and cold (10°C) stimuli

The lack of effect of morphine alone against thermal allodynia suggests, that different mechanisms mediate the abnormal reactions to noxious and innocuous thermal stimuli in neuropathic rats (Lee *et al*., 1994). Indeed, cold allodynia has been considered as a significant clinical sign of sympathetic dysfunction and is used in humans to assess sympathetically-

maintained pain (Frost *et al*., 1988). Accordingly, there is evidence that in the mononeuropathic rat, the development of allodynia-like behaviour to cold stimulation can be prevented by sympatholytic treatments or surgical sympathectomy (Perrot *et al*., 1993; Desmeules *et al*., 1995), and the α_2-adrenoceptor agonist, clonidine, is highly effective against cold stimuli (Kayser *et al*., 1995a). The nature of the abnormal interaction between the sensory and the sympathetic nervous system after peripheral nerve injury remains uncertain (Jänig & Kolzenburg, 1992). Our findings indicate that EAA, acting *via* the NMDA receptor, may play a role in this sympathetic-afferent coupling. Interestingly, recent morphological evidence suggests that lumbar postganglionic sympathetic axons express the NMDA receptor (Carlton *et al*., 1998). We can only speculate, that antagonism at these NMDA receptors, *via* a sympathetic-sensory interaction could lower the activity in the primary afferent fibre. This decrease in the afferent drive might be sufficient to restore an effect of the highest dose of morphine against cold allodynia, and presuming that similar mechanisms are involved, a dose-dependent effect of morphine against a warm (44°C) stimulus.

The effect of the combination of (+)-HA966 with morphine was not associated with any detectable sedation or motor disturbances. The present results indicate that combined systemic administration of antagonists, acting at the glycine site on the NMDA receptor, in combination with morphine may be a promising therapeutic approach in treating neuropathic as well as acute pain, with a reduced risk of undesirable side effects.

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