

## Basic Sciences

# NMDA RECEPTOR ANTAGONISTS ATTENUATE TOLERANCE INDUCED BY MORPHINE AND NERVE LIGATION IN MICE

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### ABSTRACT

The effect of NMDA (N-methyl-D-aspartate) receptor antagonists on tolerance to morphine antinociception was investigated in mice. Daily subcutaneous administration of 50 mg/kg of morphine hydrochloride for three days induced tolerance to different (3,6 and 9 mg/kg) test doses of morphine. The tolerance obtained was decreased by pretreatment of animals with single or repeated doses of competitive NMDA receptor antagonists D-(-)-2-amino-5-phosphonovaleric acid (DAP5; 0.1-0.3 mg/kg) and D-(-)-2-amino-7-phosphonoheptanoic acid (DAP7; 0.1-0.3 mg/kg).

Tolerance to morphine response was also obtained 7, 14, 21 and 30 days after unilateral sciatic nerve ligation. Single or repeated doses (0.2 mg/kg) of DAP5 or DAP7 increased the antinociceptive response induced by morphine (9 mg/kg) in the nerve ligated animals. We report that these agents attenuate the development of morphine tolerance, and increase the antinociceptive effect of morphine on sciatic neuropathic pain in mice.

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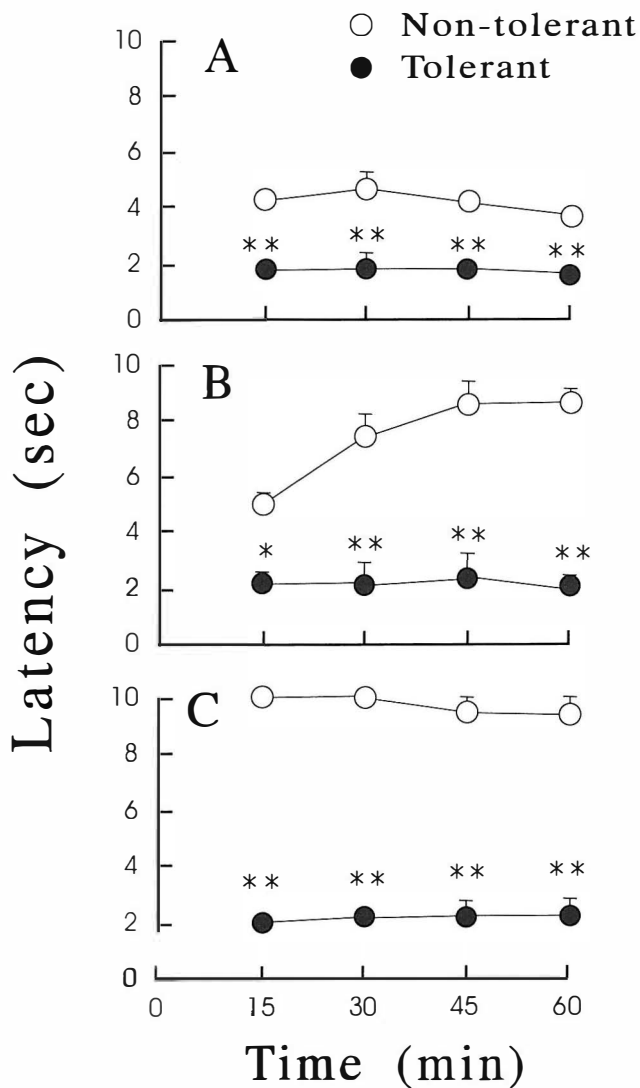
### INTRODUCTION

Opiate drugs such as morphine are widely used in the clinical management of pain. One major problem in the use of opiate analgesic drugs is the development of tolerance during their administration. The lack of efficacy against neuropathic pain, produced by diseases or damage to the nervous system, not only reduces their effectiveness but also complicates the management of patients with persistent

pain.<sup>10</sup> Both laboratory animals<sup>20,26,27</sup> and humans<sup>7</sup> can show the development of tolerance and lack of effectiveness to morphine analgesia.

In addition, nerve damage that affects peripheral nerves leads to abnormal pain states referred to as neuropathic pain. The well-established animal model of peripheral mononeuropathy produced by persistent moderate constriction of the common sciatic nerve, has been studied extensively.<sup>15,19</sup> The excitatory amino acids have been suggested to be involved in endogenous pain inhibitory mechanism(s).<sup>2</sup> Excitatory amino acid systems exert a wide variety of pharmacological effects. In particular, the NMDA receptor may be involved in neural development, long-term potentiation, kindling, learning, and memory.<sup>24</sup>

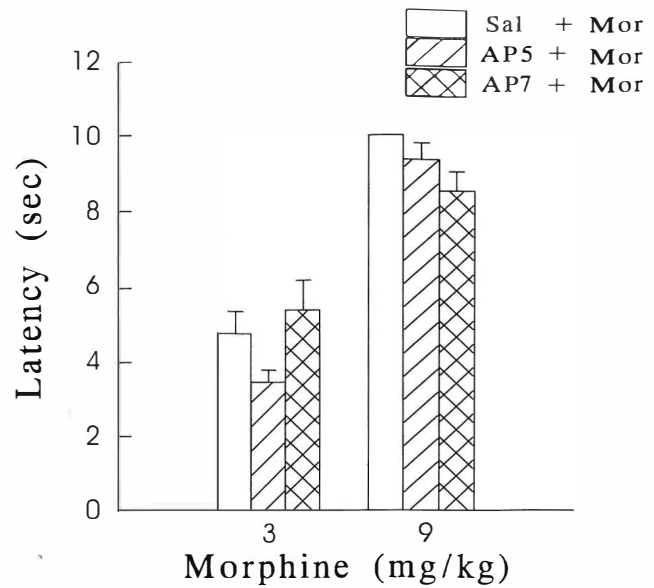
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**Fig. 1.** Comparison of antinociception in non-tolerant and tolerant mice to morphine. Mice were administered morphine 50 mg/kg daily for a period of three days in order to develop tolerance to morphine antinociception. Non-tolerant animals received only daily administration of saline instead of morphine injection. Non-tolerant or tolerant animals were injected subcutaneously (s.c.) with test doses of morphine; 3 (graph A), 6 (graph B) or 9 mg/kg (graph C). Each point is mean  $\pm$  SEM of 8 animals. \* $p < 0.01$ , \*\* $p < 0.001$ , different from the non-tolerant group.

It has been suggested that the NMDA receptors play a major role in the development of morphine tolerance.<sup>22</sup> Inhibition of morphine tolerance and dependence by NMDA receptor antagonists has been shown.<sup>24</sup> NMDA antagonists have also been shown to attenuate mechanical allodynia in some animal models of nerve injury;<sup>21</sup> therefore, EAA antagonists may provide a new treatment application.

In the present study we examined the effect of the spe-



**Fig. 2.** Effects of NMDA receptor antagonists on non-tolerant mice. The non-tolerant animals were treated (s.c.) with saline (10 mL/kg), DAP5 or DAP7 (0.2 mg/kg) 30 min prior to test doses of 3 or 9 mg/kg of morphine. Antinociception was recorded 30 min. after morphine injection. Each point is mean  $\pm$  SEM of 7 animals.

cific NMDA antagonist DAP5 and DAP7 on the development of morphine tolerance, and effectiveness of the opioid drug on neuropathic pain.

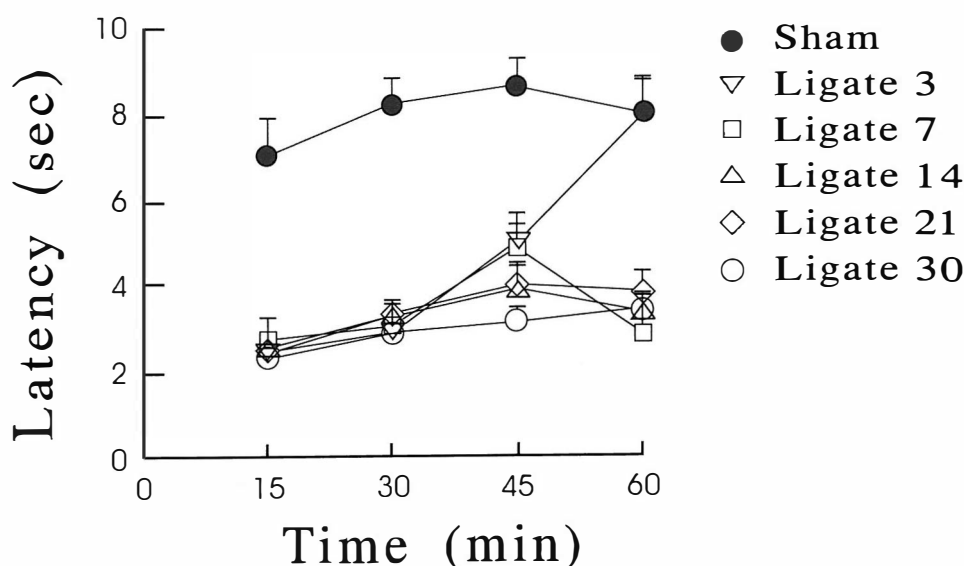
## MATERIAL AND METHODS

### Subjects

Male adult albino mice (weighing 20-25g at the beginning of experiments) were used in the experiments. The animals were housed in groups of 10 in conditions of constant temperature ( $21 \pm 2^\circ\text{C}$ ) and a light controlled room (light period, 07:00-19:00). Animals had free access to food and water except during the experiments. Each animal was used only once, and was euthanized immediately after experiments.

### Surgical procedure for nerve ligation

The animals were anesthetized with ether. After an adequate depth of anesthesia was verified by lack of response to tail pinch, an incision was made from the right sciatic notch to the distal thigh. The subcutaneous tissue was bluntly dissected under the skin to expose the biceps femoris muscle, a cut was made into the muscle at the sciatic notch, then the muscle fibers were bluntly spread, and a 2-3 mm long nerve segment was then dissected. One ligature (fine metal wire) was made around the dissected nerve. Mice in sham operation groups underwent the same surgical procedures except for sciatic nerve ligation. All animals received cephalixin pow-



**Fig. 3.** Effect of sciatic nerve-ligation on morphine antinociception in mice. Animals were either sham-operated or unilateral nerve ligated (on the right sciatic) and antinociceptive response of a test dose of morphine (9 mg/kg, s.c.) was tested 3, 7, 14, 21 and 30 days after ligation. Each point is mean  $\pm$  SEM of 7 animals. All points are significant with sham-operated animals except for the effect of morphine 3 days after ligation and 60 min after morphine injection.

der at the site of nerve ligation, in order to prevent infection.

#### Development of tolerance to morphine

Tolerance was achieved as we previously described.<sup>20,26,27</sup> Mice were treated subcutaneously (s.c.) with a daily dose of morphine (50 mg/kg) for 3 days. To assess the degree of tolerance, the antinociceptive response to different test doses of morphine (3, 6 and 9 mg/kg) was measured on the 4th day (a day after the last morphine injection).

#### Tail-flick assay

A day after tolerance induction, and on day 3, 7, 14, 21 and 30 after nerve ligation of animals, the antinociception of test doses of morphine was measured.

Antinociception was determined in the tail-flick test, wherein noxious stimulation is effected by a beam of high intensity light focused on the animal's tail with a tail-flick apparatus (model 812, Hugo Sachs Electronic, Germany). The mean response of latency period before the drug administration was 2-3 sec. in non-tolerant and 1-2 sec. in tolerant or ligated animals. A 10 sec. cut off was imposed to avoid excessive tissue damage.

#### Drugs

The following drugs were used: D-(-)-2-amino-5-phosphonovaleric acid (DAP5) and D-(-)-2-amino-7-phosphonoheptanoic acid (DAP7) (Institute of Physiologically Active Compounds, Russia), morphine hydrochloride (T & H Smith Ltd, England), and diethylether

(Merck, Germany). DAP5, DAP7 and morphine were dissolved in saline and were administered subcutaneously. The drugs were prepared immediately before use and were administered in a volume of 10 mL/kg.

#### Drug treatment

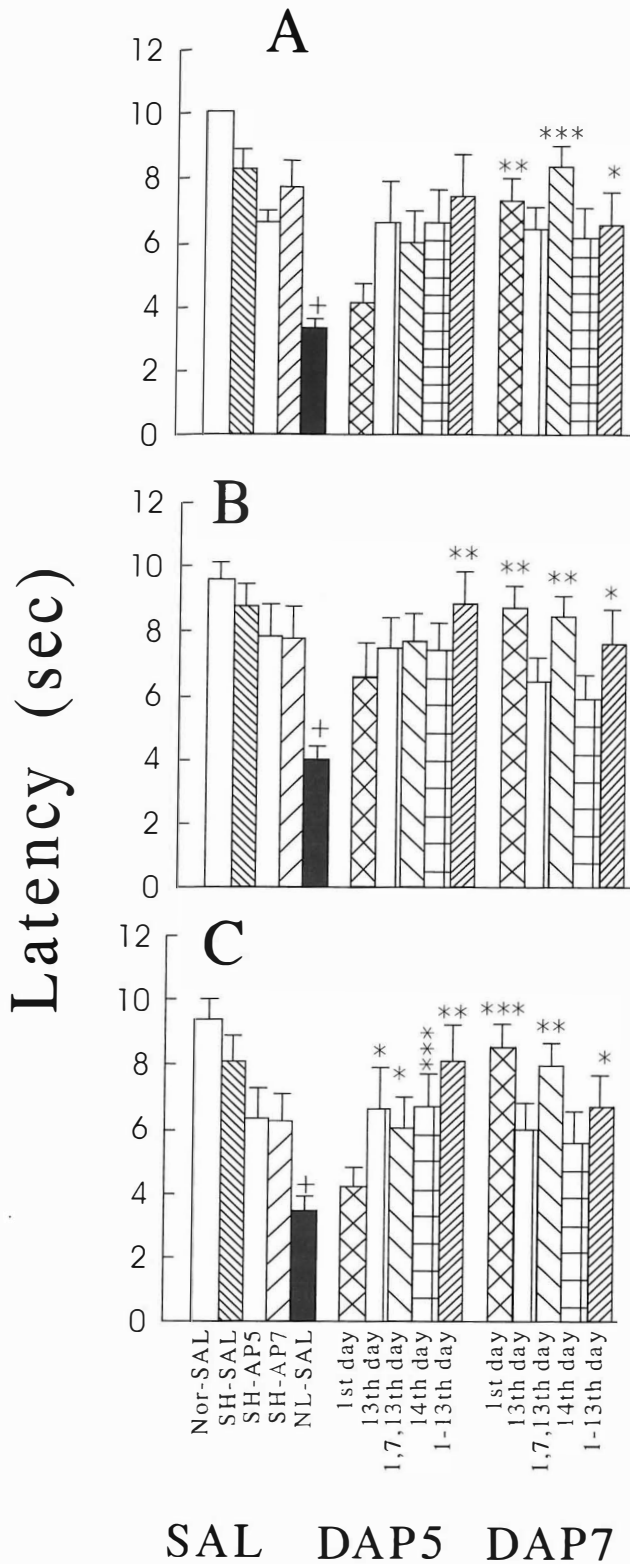
Animals in experiment 1 received a subcutaneous (s.c.) injection of morphine (50 mg/kg) once daily for three days, in order to induce tolerance to morphine antinociception, and the antinociceptive response of the test doses of opioid (3, 6 and 9 mg/kg) was measured by tail flick tested on the 4th day.

Animals in experiment 2 received daily DAP5 or DAP7 (0.1, 0.2 and 0.3 mg/kg) 30 min before a daily dose of morphine 50 mg/kg (during induction of tolerance to morphine) and a test dose of morphine (9 mg/kg) was tested on the 4th day.

Animals in experiment 3 received normal saline for three days instead of morphine, and on the 4th day (30 min prior to test doses of morphine; 3, 6 and 9 mg/kg) either DAP5 or DAP7 (0.2 mg/kg; s.c.) was injected and the response of the test doses of morphine was recorded 30 min after the opioid administration.

Animals in experiment 4 (to test the effect of morphine on nerve-ligation) were sciatic nerve-ligated and the antinociceptive response of morphine (9 mg/kg, s.c.) was tested 7, 14, 21 and 30 days after ligation.

Animals in experiment 5 were sciatic nerve-ligated and the antinociceptive response of morphine (9 mg/kg, s.c.) was tested in intact animals, sham-operated or 14 days after nerve-ligation.



**Fig. 4.** Effect of NMDA receptor antagonists on tolerance induced by sciatic nerve ligation. Intact mice (Nor), Sham-operated (SH) or nerve-ligated animals (NL) were treated (s.c.) with saline (SAL), DAP5 or DAP7 and antinociception of a test dose of morphine (9 mg/kg) was tested 14 days after nerve ligation. Antinociception was recorded 30 (A), 45 (B) and 60 min (C) after morphine administration. Each point is mean  $\pm$  SEM of 7 animals. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001, different from the nerve ligated-group. \* $p$ <0.001, different from sham-operated or intact animals.

**Statistical analysis**

Analysis of variance (ANOVA) followed by Newman-Keul's test were used to evaluate the significance of the results. A value of  $p$ <0.05 was considered significant.

**RESULTS**

**Induction of tolerance to morphine**

Daily subcutaneous (s.c.) administration of morphine hydrochloride (50 mg/kg) for three days resulted in the development of tolerance. Antinociception of test doses of morphine (3, 6 and 9 mg/kg) was recorded on the 4th day after drug injection (24 h after the last dose of morphine). The antinociceptive effect to test doses of 3 mg/kg [ $F(7, 56)=25.7, p<0.0001$ ], 6 mg/kg [ $F(7, 56)=40.9, p<0.0001$ ] and 9 mg/kg [ $F(7, 56)=193.8, p<0.0001$ ] of morphine was significantly decreased in tolerant mice (Fig. 1).

**Effects of NMDA receptor antagonists on morphine-induced tolerance**

Table I indicates the time course of development of morphine tolerance in the presence or absence of NMDA receptor antagonists. The animals were treated with daily doses of 50 mg/kg of morphine for three days in order to induce tolerance and were tested 24h after the last daily dose of the opioid. In these animals, the antinociceptive response of a test dose of 9 mg/kg of morphine was reduced. When animals were treated daily with DAP5 or DAP7 (0.1, 0.2 and 0.3 mg/kg) 30 min before daily doses of morphine (50 mg/kg), tolerance to a test dose of morphine (9 mg/kg) was reduced. The response to a test dose of morphine (9 mg/kg) was also increased when animals were administered DAP5 or DAP7 (0.2 mg/kg) on the 1st, 2nd or 3rd day of daily morphine administration (50 mg/kg) or on the 4th day, 30 min prior to a test dose of morphine injection. It is worth notice that tolerance was less prominent when NMDA receptor antagonists were administered on the 1st or 2nd day rather than the 3rd or 4th day. The difference between time course response of morphine may indicate that chronic treatment with the antagonists elicits more response.

**Effects of NMDA receptor antagonists on morphine antinociception in non-tolerant mice**

Animals received normal saline for three days instead of a daily administration of morphine, and on the 4th day (30 min before test doses of morphine; 3 and 9 mg/kg), either DAP5 or DAP7 (0.2 mg/kg, s.c.) was administered and the response of test doses of morphine was recorded. ANOVA did not show any difference between the response induced in saline-treated with those obtained in NMDA receptor antagonist-treated animals, for the test doses of 3 [ $F(2, 18)=2.5, p>0.05$ ] or 9 mg/kg [ $F(2, 18)=3.5, p>0.05$ ] (Fig. 2).

### Antinociceptive effect of morphine in sciatic nerve-ligated mice

Fig. 3 indicates antinociception of morphine in sham-operated and nerve ligated animals. The antinociceptive response of morphine was decreased significantly in the nerve ligated as compared with the sham-operated animals. Consistently, antinociception induced by the test dose of morphine was decreased 3, 7, 14, 21 and 30 days after sciatic nerve ligation [ $F(23, 168)=20.2, p<0.0001$ ]. Further analysis showed that the effect of morphine after 3 days ligation tends to decrease 45 min and become abolished 60 min after administration of a test dose of morphine. Animals which had nerve ligation for 14 days, and a test dose of morphine (9 mg/kg) were used for the rest of the experiments.

### Effects of NMDA receptor antagonists on morphine-induced antinociception in sciatic nerve ligated animals

Fig. 4 presents the effects of DAP5 and DAP7 on sham-operated and nerve-ligated animals. ANOVA indicated a difference between antinociception induced by a test dose of morphine (9 mg/kg) in sham-operated and nerve-ligated animals, 30 min [ $F(4, 30)=26, p<0.0001$ ], 45 min [ $F(4, 30)=7.8, p<0.001$ ] or 60 min [ $F(4, 30)=8.7, p<0.0001$ ] after morphine administration. Further analysis showed that sciatic nerve ligation reduced the antinociception of morphine as compared with normal or sham-operated groups. There was no difference between normal and sham-operated animals, moreover DAP5 and DAP7 did not elicit any effect on sham-operated animals. ANOVA also indicated a difference between the response of a test dose of morphine in the nerve-ligated animals which received saline with those which received single or multiple doses of DAP5, 30 min [ $F(5, 36)=2.7, p<0.05$ ], 45 min [ $F(5, 36)=3.4, p<0.05$ ] and 60 min [ $F(5, 36)=5.8, p<0.001$ ] after morphine injection. Further analysis showed that DAP5 is capable of reversing nerve ligation-reduced morphine antinociception. However, analysis showed that response to DAP5 was maximally induced 45 min after morphine administration.

In the same way also reversed ligation decreased morphine antinociception, 30 min [ $F(5, 36)=5, p<0.01$ ], 45 min [ $F(5, 36)=5.7, p<0.001$ ] and 60 min [ $F(5, 36)=5.2, p<0.01$ ] after injection of a test dose of morphine. Analysis indicated that the response of DAP7 starts 15 min after morphine injection.

## DISCUSSION

The measurement of antinociception by the tail-flick test may be predominantly spinally controlled<sup>8,9</sup> and activation of spinal cord NMDA receptors has been shown to be critical for tolerance to the antinociceptive effects of morphine.<sup>12,16,18,23,24</sup> In the present study, the effects of NMDA receptor antagonists on tolerance to morphine antinociception induced by repeated administration of mor-

phine and also the effects of the antagonists on hyperalgesia induced by ligation have been investigated in the tail-flick test. Our data showed that daily administration of morphine (50 mg/kg, subcutaneously) for a period of 3 days to mice induced tolerance to morphine antinociception in the tail-flick test. This is in agreement with our previous studies.<sup>20,26,27</sup> The results indicate that NMDA receptor antagonists decrease the development of morphine tolerance and increased morphine antinociception in nerve ligated-animals.

The present study indicates that low doses of competitive NMDA receptor antagonists DAP5<sup>13</sup> or DAP7<sup>11</sup> reduce morphine tolerance. The data are in agreement with that obtained by others<sup>24,25</sup> and may support the suggestion that the NMDA receptor mechanism may have an important role in opiate tolerance.<sup>23</sup>

Our data also indicate that administration of the NMDA antagonists DAP5 and DAP7, either during development of tolerance or in the animals in which tolerance had already been acquired, increases morphine's response. However, the response to DAP5 was maximally induced 45 min after morphine administration, while that of DAP7 began 15 min after morphine injection. Thus it appears that the effect of DAP7 begins earlier than DAP5, possibly due to early absorption of the drug. The lack of increase in morphine analgesia in non-tolerant mice by the NMDA receptor antagonists DAP5 and DAP7, which is supported by other investigators<sup>17</sup> may confirm this conclusion that the antagonists increased opioid analgesia in tolerant animals by inhibition of the development or by inhibition of the expression of morphine tolerance, possibly-through blockade of NMDA receptor antagonists. Development of morphine tolerance has been shown to be related to NMDA receptor activation and subsequent intracellular changes, and the expression of tolerance is likely due to the decreased effectiveness of mu opioid receptors to morphine.<sup>15</sup> The electrophysiological link between mu receptor occupancy and an increase in  $Ca^{++}$  conductance by ion channels regulated by NMDA receptors has been proposed.<sup>3</sup> There is also a suggestion that NMDA receptor-activated, calcium-dependent processes may be involved in the development of opiate tolerance.<sup>25</sup> Because of the well-described role of calcium ions in NMDA receptor-mediated neural and behavioural plasticity,<sup>4,5</sup> the possibility may exist that the NMDA receptor antagonists DAP5 and DAP7 inhibit the development of opiate tolerance through such a mechanism. Whether this mechanism is involved in the response of acute and/or subchronic administration of the antagonists requires further experiments.

Our data also showed that sciatic nerve-ligation reduces the antinociceptive effect of morphine. This was in agreement with recent studies using an animal model of neuropathic pain.<sup>1,23</sup> There nerve induces hyperalgesia in animals.<sup>19</sup> The release of NMDA in the presence of excitatory activity resulting from

## NMDA Receptor Antagonists Attenuate Tolerance

**Table I.** Effects of NMDA receptor antagonists on tolerance induced to morphine antinociception.

Treatment (mg/kg)	Day of Treatment	Latency after morphine (sec)			
		15 min	30 min	45 min	60 min
Saline (10 mL/kg)		2.03±0.05	2.20±0.06	2.24±0.03	2.24±0.11
DAP5 0.1	(1,2,3rd)	3.01±0.22	5.40±0.6	5.86±0.5*	3.67±0.34
DAP5 0.2	(1,2,3rd)	4.16±0.61	5.88±0.88	7.47±0.87**	7.27±0.80**
DAP5 0.3	(1,2,3rd)	3.60±0.59	6.54±1.25*	6.79±0.99*	6.74±1.01**
DAP5 0.2	(1st)	6.39±1.31*	7.44±1.08*	7.64±0.98**	7.37±0.76**
	(2nd)	6.76±0.77**	8.87±0.57**	8.73±0.78**	6.86±0.73**
	(3rd)	4.09±0.41	5.84±0.85	7.64±0.97**	7.66±0.73**
	(4th)	4.60±0.91	5.26±0.93	6.80±0.65*	6.46±0.77*
DAP7 0.1	(1,2,3rd)	3.51±0.69	5.60±0.86*	5.71±0.72*	3.30±0.24
DAP7 0.2	(1,2,3rd)	2.94±0.14	3.36±0.25	3.84±0.65	5.59±1.22*
DAP7 0.3	(1,2,3rd)	3.75±0.57	6.00±0.79*	7.20±0.88**	9.35±0.38**
DAP7 0.2	(1st)	6.91±1.11**	9.59±0.41**	9.33±0.49**	8.17±0.80**
	(2nd)	6.90±1.19**	7.70±1.01**	7.90±0.64**	6.34±0.73**
	(3rd)	4.14±0.29	8.00±0.64**	8.71±0.41**	9.03±0.44**
	(4th)	4.41±0.82	9.00±0.45**	10.0±0.00**	9.10±0.66**

Mice received a daily injection of 50 mg/kg of morphine subcutaneously for three days, in order to induce tolerance to morphine. Different groups of the animals subjected to tolerance were injected with either saline, DAP5 or DAP7 on the 1st, 2nd or 3rd day or for three days 30 min prior to daily injection of morphine (50 mg/kg) during tolerance induction, or 30 min before a test dose of morphine (9 mg/kg). The antinociceptive response to a test dose of morphine (9 mg/kg) was recorded 15, 30, 45 and 60 min after morphine injection on the 4th day (a day after tolerance to morphine). Each value is mean ± SEM of 8 mice. \* $p < 0.05$ , \*\* $p < 0.01$ , different from control group.

nerve injury yields central sensitivity, manifested as allodynia and hyperalgesia, and presumed development of tolerance.<sup>6-14</sup> The reduced morphine antinociception induced by nerve injury may share a mechanism similar to that of morphine tolerance. The basis of interaction between mechanisms of reduced morphine response in morphine tolerance and nerve injury is believed to be related to common neural substrates and the site of action involved in both phenomena.<sup>15</sup>

The present results showed that daily administration of the specific NMDA receptor antagonists DAP5 and DAP7 can increase morphine induced antinociception in neuropathic animals. If reduction of the antinociceptive effect of morphine develops in animals with nerve injury or morphine tolerance results from the activation of spinal cord NMDA receptors and consequent intracellular cascades, this view is strongly supported by our observation that (1) both the development of morphine tolerance and reduced morphine antinociception in nerve injured mice could be pre-

vented by daily treatment with selective NMDA receptor antagonists, (2) three, two or even a single injection of the NMDA antagonists prevented the development of tolerance and reduction of morphine antinociception in nerve-injured mice, and (3) a single injection of NMDA receptor antagonists in tolerant animals (on the 4th day, 30 min before a test dose of morphine) or in neuropathic animals (on the 14th day) could increase morphine induced analgesia. It means that the expression of morphine tolerance and neuropathic pain could be reversed by a single dose of DAP5 or DAP7, and this emphasized the existence of a common cellular and intracellular mechanism for both phenomena. This commonality has not been shown by other non-competitive NMDA receptor antagonists<sup>15</sup> and the reason(s) may be due to involvement of different subtypes of NMDA receptors. These findings would help to develop new opioid treatment approaches for improved treatment of at least some pathophysiological pain syndromes and opiate tolerance.

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