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European Journal of Pharmacology 409 (2000) 279–289

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Possible mechanisms of action in melatonin reversal of morphine tolerance and dependence in mice

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Received 6 July 2000; received in revised form 26 October 2000; accepted 31 October 2000

Abstract

In our earlier study, we reported the ability of melatonin to reverse the development of morphine tolerance and dependence in mice. In the present study, we attempted to analyse the possible involvement of putative melatonin receptors, central and peripheral benzodiazepine receptors and the nitric oxide (NO) system in the mechanism of melatonin reversal of morphine tolerance and dependence in mice. Co-administration of L-N^G-nitro arginine methyl ester (L-NAME) or melatonin with morphine during the induction phase (days 1–9) delayed the development of tolerance to the anti-nociceptive action of morphine and also reversed naloxone precipitated withdrawal jumpings. L-arginine administration during the induction phase enhanced the development of tolerance to the anti-nociceptive effect of morphine but had no effect on the naloxone-precipitated withdrawal response. During the expression phase (day 10), acute administration of melatonin or L-NAME reversed, whereas L-arginine facilitated, naloxone-precipitated withdrawal jumping in morphine-tolerant mice, but none of these drugs affected the nociceptive threshold in morphine-tolerant mice. Further, co-administration of melatonin or L-NAME with L-arginine during the induction phase antagonized later the effects on the development of morphine tolerance. Also, prior administration of melatonin or L-NAME reversed the L-arginine potentiation of naloxone-precipitated withdrawal jumping in morphine tolerant mice. Among the antagonists for putative melatonin receptors studied, neither luzindole (melatonin MT_1 and MT_2 receptor antagonist) nor prazosin (melatonin MT_3 receptor antagonist) antagonized the melatonin reversal of morphine tolerance and dependence. 1-(2-Chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195), a peripheral but not central benzodiazepine receptor antagonist, flumazenil, partially antagonized the melatonin reversal of naloxone-precipitated withdrawal jumping in morphine-dependent mice, but had no effect on the reversal of morphine tolerance induced by melatonin. Overall, the present observations suggest that the melatonin-induced reversal of morphine tolerance and dependence may involve its ability to suppress nitric oxide synthase (NOS) activity. Further, the melatonin-induced reversal of morphine tolerance and dependence is not mediated through its actions via putative melatonin receptors. The agonistic activity of melatonin towards peripheral benzodiazepine receptors may partially contribute to the suppression of morphine dependence but not to the reversal of tolerance to the analgesic activity of morphine.

Keywords: Morphine tolerance; Morphine dependence; Melatonin MT_1 receptor; Melatonin MT_2 receptor; Melatonin MT_3 receptor; Benzodiazepine receptor; Central/peripheral; Nitric oxide (NO); Tail-flick latency; Withdrawal jump

1. Introduction

The mechanisms involved in the development and expression of opioid tolerance and dependence remain unclear despite a great deal of research. It has been postulated that opioid tolerance and dependence may be distinct phenomena, developing independently of each other (Bhargava, 1994). Moreover, both phenomena have two distinct phases, the induction phase, during which the processes underlying the observed changes occur, and the expression phase, during which changes occur after cessation of drug administration (Dambisya and Lee, 1996). A wide range of neurotransmitters or neuromodulators play a role in the development of tolerance and dependence to opioids, for example, serotonin, norepinephrine, dopamine, γ-aminobutyric acid (GABA)-benzodiazepine, adenosine, excitatory amino acids, nitric oxide (NO), acetylcholine, oxytocin and vasopressin, etc. (Bhargava, 1994). In many instances, modulation of the functions of these neurotransmitters or neuromodulators may play a different role in the

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00849-9

induction and expression of morphine tolerance and dependence (Bhargava, 1994; Dambisya and Lee, 1996). So it is necessary to study the effect of test drugs on both the phases of morphine tolerance and dependence.

In our earlier study, we showed that the pineal hormone melatonin reversed the development of tolerance and dependence to morphine when administered during the induction phase, whereas it suppressed the withdrawal response but had no effect on the nociceptive threshold when administered during the expression phase of morphine tolerance and dependence. Also, the melatonin-induced reversal of morphine dependence but not tolerance was partially antagonized by 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195), a peripheral benzodiazepine receptor antagonist (Raghavendra and Kulkarni, 1999). It appears from this study that multiple mechanisms may contribute to the reversal of morphine tolerance and dependence by melatonin. Melatonin, by acting through its own plasma membrane receptors, facilitates inhibitory GABAergic neurotransmitter functions (Kopp et al., 1999; Wan et al., 1999), antagonizes 5-$HT_{2A/2C}$ receptor-mediated behavioral responses (Eison et al., 1995; Drago et al., 1999; Raghavendra and Kulkarni, 2000) and affects cholinergic neurotransmission in the nucleus accumbens (Paredes et al., 1999). Besides these actions, at a cellular level melatonin suppresses nitric oxide synthase (NOS) activity via complex formation with cytoplasmic calmodulin in brain regions concerned with motor, autonomic and vegetative functions (Pozo et al., 1997; Bettahi et al., 1996). It is reported that modulators of GABA-benzodiazepine, 5-HT_2, the cholinergic system and NOS activity differentially regulate morphine tolerance and dependence (Bhargava, 1994; Dambisya and Lee, 1996). Recently, it has been shown that the effect of melatonin on cocaine-induced behavioral sensitization in rats partially resembled that of NOS inhibitors (Sircar, 2000). So, it is reasonable to study the influence of these systems on the melatonin-induced reversal of morphine tolerance and dependence. The present study was designed to evaluate whether melatonin receptor antagonists (luzindole or prazosin), central/peripheral benzodiazepine antagonists (flumazenil or PK11195) or modulators of NO systems (L-N^G-nitro arginine methyl ester (L-NAME) or L-arginine) affect the melatonin-induced reversal of morphine tolerance and dependence.

2. Materials and methods

2.1. Animals

Male Balb/c mice weighing 25 ± 2 g were obtained from the Central Animal House, Panjab University, Chandigarh. The mice were kept under a 13/11-h light/dark cycle (lights on at 0600 h) and standard laboratory conditions with food and water being continuously available except during the experiment. The experimental protocol was approved by the Institutional Animal Ethics Committee. All testing was done between 0900 and 1200 h.

2.2. Induction of morphine tolerance and dependence

Tolerance to and dependence on morphine were induced in mice by repeated injection of morphine sulphate (10 mg/kg, s.c.) twice daily at 0900 and 1700 h for 9 days as described previously (Reddy and Kulkarni, 1997; Raghavendra and Kulkarni, 1999).

2.3. Assessment of morphine tolerance and dependence

The loss of the antinociceptive effects of morphine after repeated administration in the tail-flick assay was used, with modifications as described in detail elsewhere (Reddy and Kulkarni, 1997; Raghavendra and Kulkarni, 1999). A pilot study showed that the antinociceptive effect of morphine (10 mg/kg, twice daily) declined after the 3rd day and that complete tolerance was observed on the 9th or 10th day after repeated injection of morphine. We subsequently used this dose regimen of morphine to study the effect of test drugs on the development of tolerance to the antinociceptive effect. A minimum of three trials were recorded for each mouse 45 min after morphine injection on days 1, 3, 6 and 9 by tail-flick test. Development of physical dependence to morphine was assessed by counting the number of naloxone-precipitated withdrawal jumps. Immediately after the tail-flick test on day 10, naloxone (2 mg/kg, i.p.) was injected and the animals were individually placed in a plexiglass box ($45 \times 30 \times 30$ cm) and observed for withdrawal jumping for a period of 20 min.

2.4. Treatment schedule

For the assessment of the effects of various agents on the induction of morphine tolerance and dependence, the test drugs were co-administered with morphine throughout the induction (days 1–9) period; on day 10 only morphine was administered. For the assessment of the effects of the various drugs on the expression of tolerance and dependence, animals that had received only morphine during the induction phase (days 1–9) were acutely treated with test drugs along with morphine on 10th day.

2.5. Drugs

Morphine sulphate (Government Analytical Laboratory, Chandigarh, India), melatonin (Morepen Laboratory, Parwano, India) in 0.5% ethanol–water, PK11195 (RBI, Natick, MA, USA) and flumazenil (F. Hoffmann-La Roche and Co., Basel, Switzerland) in 1% dimethyl sulphoxide–water, L-arginine (Loba Chemi, Bombay, India) and L-N^G-nitro arginine methyl ester (L-NAME) (Sigma, MO,

USA) in saline, luzindole and prazosin (Sigma) in 1% Tween 20 were used in the present study. All the test drugs were administered intraperitoneally 45 min before testing tail-flick latency or naloxone (Sigma; 2 mg/kg, i.p.) challenge.

2.6. Statistical analysis

All results are expressed as means ± S.E.M. The data were analysed by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test. Differences with $P < 0.05$ between experimental groups at each point were considered statistically significant.

3. Results

3.1. Effect of chronic treatment of melatonin, L-NAME or L-arginine on morphine-induced nociception

Chronic administration (twice daily × 9 days) of melatonin (5 mg/kg), L-NAME (10 mg/kg) or L-arginine (20 mg/kg) had no effect on the anti-nociception induced by an acutely administered submaximal dose of morphine (2.5 mg/kg). In none of the animals treated with these agents alone did the nociceptive threshold change compared to that of the vehicle-treated group (Table 1).

3.2. Effect of melatonin on induction of tolerance and dependence to morphine

Mice receiving the chronic treatment with morphine (10 mg/kg) showed a maximal anti-nociceptive response on days 1 and 3 of treatment. However, the animals developed tolerance to the anti-nociceptive response as the reaction time gradually decreased on the 6th day and reached the baseline latency by day 9 of testing. Chronic treatment with melatonin (1–5 mg/kg) along with morphine reversed the development of tolerance to the analgesic effect of morphine (Fig. 1). On the 10th day of testing, when the melatonin treatment had been stopped, mice treated with the lower dose of melatonin (1 mg/kg)

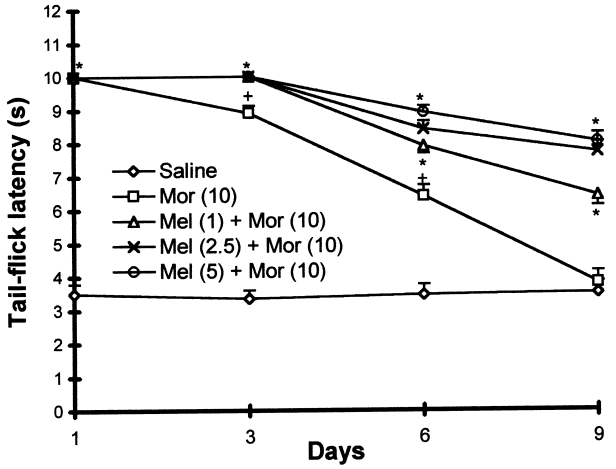

Fig. 1. Effect of chronic administration of melatonin on the development of tolerance to the analgesic effect of morphine in mice. Melatonin (1–5 mg/kg, i.p.) was administered concomitantly with morphine (10 mg/kg, s.c.) for days 1–9. The analgesic effect was recorded on the 1st, 3rd, 6th and 9th days, 45 min after morphine injection. Each point represents the means ± S.E.M. ($n = 6–8$). $^{+}P < 0.05$ vs. saline treated group; $^{*}P < 0.05$ vs. morphine alone treated group (Duncan's new multiple range test).

during induction phase failed to retain an analgesic response to morphine. However, animals treated with higher doses of melatonin (2.5 and 5 mg/kg) during the induction phase showed significant tolerance to the analgesic action of morphine on 10th day (Table 2). At the dose level studied, chronic administration of the putative melatonin receptor antagonists luzindole (2.5 and 5 mg/kg) or prazosin (2.5 and 5 mg/kg) and the central or peripheral benzodiazepine receptor antagonists, flumazenil (1 and 2 mg/kg) or PK11195 (1 and 2 mg/kg), respectively, failed to modulate the nociceptive threshold of animals or the development of morphine tolerance during the induction phase (data not shown). Also, prior administration of these drugs failed to antagonize the melatonin (2.5 mg/kg)-induced reversal of the development of tolerance to morphine (Figs. 2 and 3). Concomitant administration of these drugs with melatonin during the induction phase failed to antagonize the melatonin-induced reversal of morphine tolerance as observed on the 10th day (Table 2).

The effects of co-administration of melatonin on the induction of morphine dependence are summarized in Table 2. Concurrent administration of melatonin (1–5 mg/kg) with morphine during the induction phase attenuated naloxone (2 mg/kg)-precipitated withdrawal jumping. Administration of PK11195 (1 and 2 mg/kg) with morphine had no effect on the induction of opioid dependence, but its co-administration with melatonin (2.5 mg/kg) significantly reversed the melatonin suppressed withdrawal jumping. However, the reversal of the effect of melatonin on morphine dependence by PK11195 was partial, since both doses of the antagonist used had a similar effect and failed to antagonize melatonin effect completely. Co-administra-

Table 1
Modification of morphine (2.5 mg/kg)-induced antinociception by chronic administration melatonin, L-NAME or L-arginine

Chronic treatment (mg/kg) (twice daily × 9 days)	Tail-flick latency (s)	
	Before morphine	After morphine
Control	3.10 ± 0.52	6.28 ± 0.68
Melatonin (5)	3.27 ± 0.41	6.14 ± 0.74
L-NAME (10)	3.34 ± 0.38	6.07 ± 0.54
L-arginine (20)	2.90 ± 0.56	6.35 ± 0.68

Values are means ± S.E.M. ($n = 6–8$). ANOVA values are $F(3,21) = 0.36$ and $F(3,21) = 0.56$ for before and after morphine (2.5 mg/kg) treatment, respectively.

Table 2
Effect of melatonin (MEL) and its combination with luzindole (LUZ), prazosin (PRZ), PK11195 or flumazenil (FLU) on the induction and expression of morphine tolerance and physical dependence

Treatment	Induction		Expression	
	Tail-flick latency (s)	Withdrawal jumps/20 min	Tail-flick latency (s)	Withdrawal jumps/20 min
Control	3.47 ± 0.21	19.6 ± 1.5	3.47 ± 0.21	19.6 ± 1.5
MEL (1)	4.35 ± 0.33	4.5 ± 0.9[a]	3.38 ± 0.24	17.0 ± 1.0
MEL (2.5)	7.06 ± 0.28[a]	3.6 ± 0.8[a]	3.40 ± 0.25	7.3 ± 1.5[a]
MEL (5)	7.72 ± 0.28[a]	3.8 ± 0.5[a]	3.30 ± 0.22	4.0 ± 0.7[a]
LUZ (2.5) + MEL (2.5)	7.35 ± 0.34[a]	3.3 ± 0.6[a]	–	7.8 ± 1.4[a]
LUZ (5) + MEL (2.5)	6.88 ± 0.36[a]	4.5 ± 0.8[a]	–	8.4 ± 1.4[a]
PRZ (2.5) + MEL (2.5)	7.2 ± 0.31[a]	3.8 ± 0.8[a]	–	8.0 ± 1.1[a]
PRZ (5) + MEL (2.5)	6.82 ± 0.27[a]	3.5 ± 0.6[a]	–	8.4 ± 1.4[a]
PK11195 (1) + MEL (2.5)	7.27 ± 0.32[a]	9.5 ± 1.0[b]	–	13.5 ± 0.9[b]
PK11195 (2) + MEL (2.5)	6.78 ± 0.28[a]	10.0 ± 1.0[b]	–	14.0 ± 1.0[b]
FLU (1) + MEL (2.5)	6.97 ± 0.31[a]	4.0 ± 1.0[a]	–	6.7 ± 0.9[a]
FLU (2) + MEL (2.5)	6.9 ± 0.30[a]	4.3 ± 0.5[a]	–	7.7 ± 0.9[a]

All the animals used in the present study were chronically treated with morphine (10 mg/kg. twice daily) for 9 days. On the 10th day, 45 min after morphine (10 mg/kg) administration, the tail-flick latency of the animals was recorded and then they were treated with naloxone (2 mg/kg) to access physical dependence. The treatment schedule to evaluate the effect of test drugs on induction or expression of morphine tolerance is as explained in Materials and methods. Values are means ± S.E.M. ($n = 6–8$).

[a] $P < 0.05$ vs. control group.

[b] $P < 0.05$ vs. melatonin (2.5 mg/kg)-treated group (Duncan's multiple range test).

tion of flumazenil (1 and 2 mg/kg), luzindole (2.5 and 5 mg/kg) or prazosin (2.5 and 5 mg/kg) with melatonin (2.5 mg/kg) during the induction phase had no effect on the melatonin reversal of morphine dependence.

3.3. Effect of melatonin on expression of morphine tolerance and dependence

The expression of morphine tolerance was not affected by acute treatment with melatonin (1–5 mg/kg), but its administration along with morphine on the 10th day attenuated the expression of physical dependence in a dose-dependent manner. At the dose level studied, acute administration of luzindole (2.5 and 5 mg/kg), prazosin (2.5 and 5 mg/kg), flumazenil (1 and 2 mg/kg) or PK11195 (1 and 2 mg/kg) during the expression phase failed to modulate the nociceptive threshold in morphine tolerant mice. Also these drugs did not affect the expression of morphine dependence (data not shown). Co-administration of PK11195 (1 and 2.5 mg/kg) with melatonin (2.5 mg/kg) during the expression phase partially antagonized the melatonin attenuation of naloxone-precipitated withdrawal

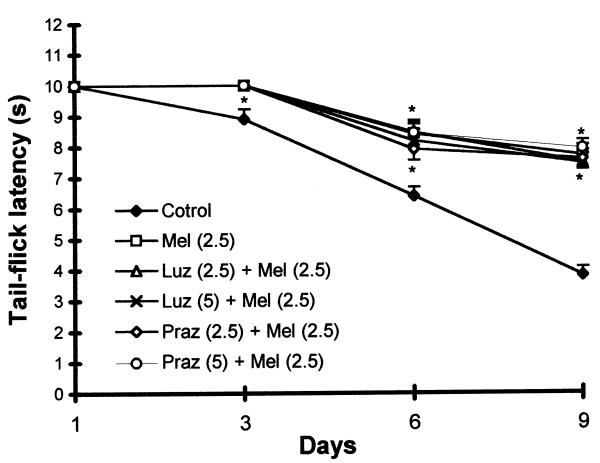

Fig. 2. Effect of the MT_1 and MT_2 or MT_3 receptor antagonist luzindole or prazosin, respectively, on the melatonin reversal of the development of tolerance to morphine. Luzindole (2.5 and 5 mg/kg, i.p.) or prazosin (2.5 and 5 mg/kg, i.p.) was administered 5 min prior to melatonin (2.5 mg/kg, i.p.). Each point represents the means ± S.E.M. ($n = 6–8$). * $P <$ 0.05 vs. control (morphine alone) group (Duncan's new multiple range test).

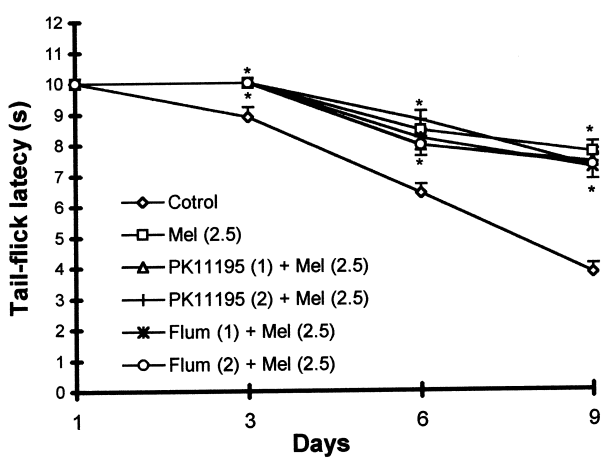

Fig. 3. Effect of the central or peripheral benzodiazepine receptor antagonist flumazenil or PK11195, respectively, on the melatonin reversal of the development of tolerance to morphine. Flumazenil (1 and 2 mg/kg, i.p.) or PK11195 (1 and 2 mg/kg, i.p.) was administered 5 min prior to melatonin (2.5 mg/kg, i.p.). Each point represents the mean ± S.E.M. ($n = 6–8$). * $P < 0.05$ vs. control (morphine alone) group (Duncan's new multiple range test).

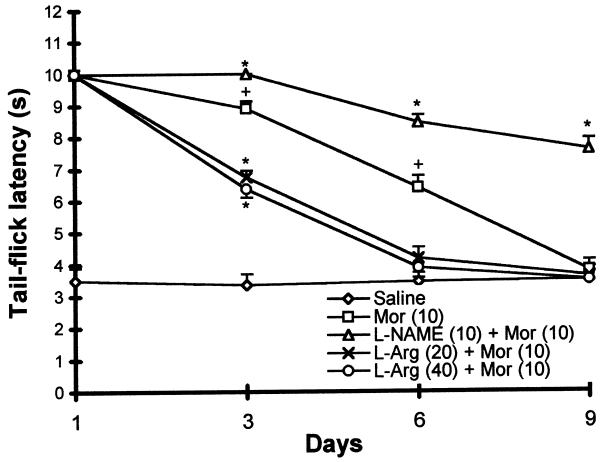

Fig. 4. Effect of chronic administration of L-NAME or L-arginine (L-Arg) on the development of tolerance to the analgesic effect of morphine in mice. L-NAME (10 mg/kg, i.p.) or L-arginine (20 and 40 mg/kg, i.p.) was administered concomitantly with morphine (10 mg/kg, s.c.) for days 1–9. The analgesic effect was recorded on the 1st, 3rd, 6th and 9th days, 45 min after morphine injection. Each point represents the mean ± S.E.M. ($n = 6$–8). $^{+}P < 0.05$ vs. saline-treated group; $^{*}P < 0.05$ vs. morphine-treated group (Duncan's new multiple range test).

jumping. Whereas co-administration of flumazenil (1 and 2 mg/kg), luzindole (2.5 or 5 mg/kg) or prazosin (2.5 and 5 mg/kg) with melatonin (2.5 mg/kg) during the expression phase failed to antagonize the melatonin reversal of morphine dependence in mice (Table 2).

3.4. Effect of L-NAME or L-arginine on the induction of morphine tolerance and dependence

Fig. 4 shows the time (in days) course of the development of morphine tolerance in the presence of L-NAME or L-arginine. Chronic administration of L-NAME (10 mg/kg) with morphine (10 mg/kg) delayed the development of tolerance to the analgesic action of morphine, whereas animals treated chronically with L-arginine (20 and 40 mg/kg) during the induction phase developed tolerance to the analgesic effect of morphine more rapidly. In L-arginine-treated animals, a significant decrease in the analgesic effect of morphine was observed on the 3rd day, and complete tolerance was observed by the 6th day. As summarized in Fig. 5A, administration of L-NAME (10 mg/kg) during the induction phase significantly reversed the development of tolerance to the analgesic effect of morphine. Although, mice treated chronically with L-arginine (20 and 40 mg/kg) and morphine showed an early development of tolerance, on the 10th day of testing L-arginine failed to show any significant effect on the nociceptive threshold as compared to morphine alone (control). Also concurrent administration of L-NAME with morphine during the induction phase led to less marked naloxone precipitated withdrawal jumping compared to chronic morphine alone (control). Although co-administration of L-arginine (20 and 40 mg/kg) with morphine during the induction phase increased the scores for nalox-

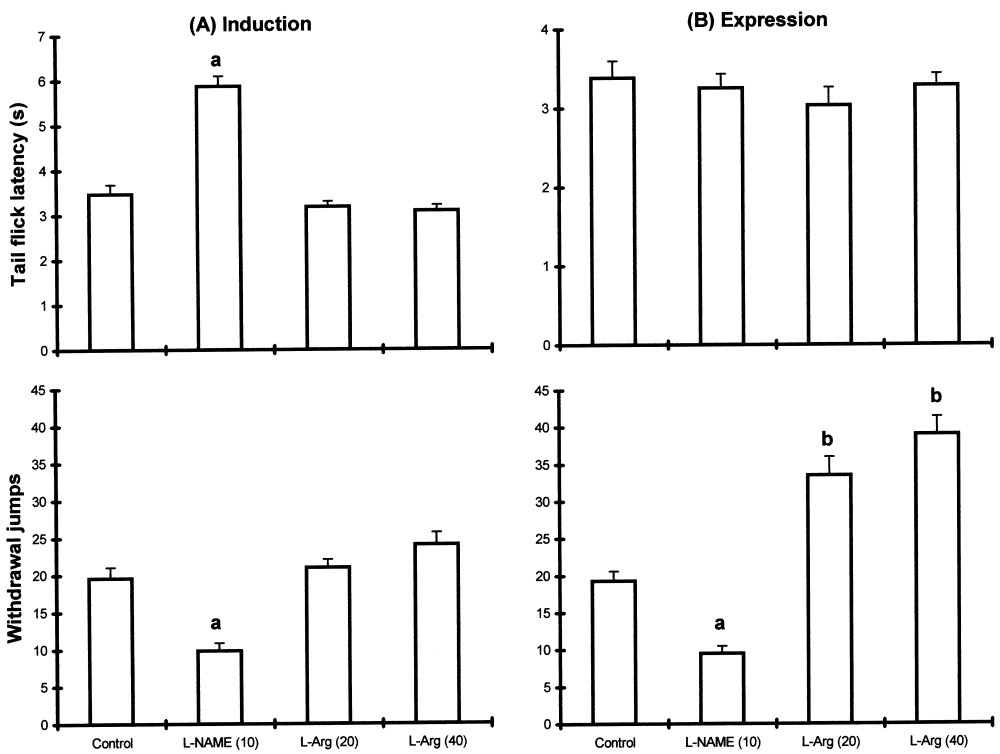

Fig. 5. Effect of (A) chronic (induction phase) or (B) acute (expression phase) administration of L-NAME or L-arginine (L-Arg) on morphine tolerance and dependence in mice. The treatment schedule to evaluate the effect of L-arginine (20 and 40 mg/kg) or L-NAME (10 mg/kg) on the induction and expression phase of morphine tolerance and dependence is explained in Materials and methods. Each bar represents the mean ± S.E.M. ($n = 6$–8). $^{a}P < 0.05$ vs. morphine (10 mg/kg)-treated group (control group) (Duncan's new multiple range test).

one-precipitated withdrawal jumping, the increase was not statistically significant compared to that of the control group.

3.5. Effect of L-NAME or L-arginine on the expression of morphine tolerance and dependence

Acute administration of L-NAME (10 mg/kg) or L-arginine (20 and 40 mg/kg) during the expression phase did not modify the tolerance to the analgesic effect. Co-administration of L-NAME (10 mg/kg) with morphine during the expression phase reduced the severity of naloxone-precipitated withdrawal jumping. While acute administration of L-arginine (20 and 40 mg/kg) during the expression phase significantly enhanced the withdrawal response (Fig. 5B).

3.6. Effect of co-administration of L-arginine with L-NAME or melatonin on the induction of morphine tolerance and dependence

Fig. 6 shows the time (in days) course of the development of morphine tolerance in the presence of co-administration of L-arginine with L-NAME or melatonin. Co-administration of L-NAME (10 mg/kg) with L-arginine (20 mg/kg) delayed the development of tolerance to morphine. But on increasing the dose of L-arginine to 40 mg/kg, not only was the L-NAME (10 mg/kg) effect reversed, but also animals developed complete tolerance to the analgesic effect of morphine by the 6th day. Similar to L-NAME, melatonin (2.5 mg/kg) retained its effect of

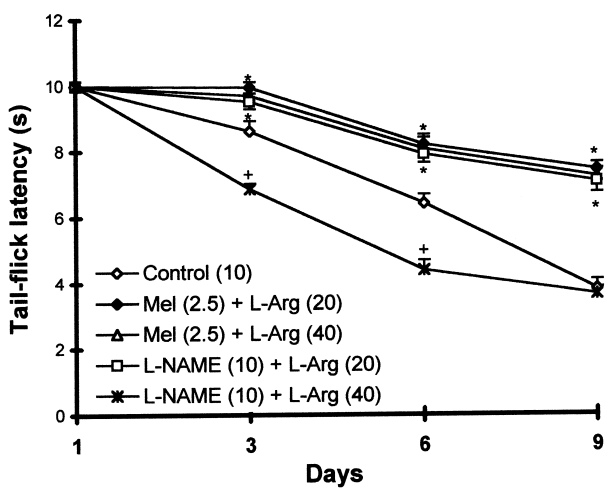

Fig. 6. Effect of co-administration of L-arginine with L-NAME or melatonin on the development of morphine tolerance. L-arginine (L-Arg; 20 and 40 mg/kg, i.p.) and L-NAME (10 mg/kg, i.p.) or L-arginine (L-Arg; 20 and 40 mg/kg, i.p.) and melatonin (2.5 mg/kg, i.p.) were co-administered along with morphine (10 mg/kg) for a period of 9 days. The analgesic effect was recorded on the 1st, 3rd, 6th and 9th days, 45 min after morphine injection. Each point represents the mean ± S.E.M. (n = 6–8). * Significantly greater, and + significantly less than the morphine-treated group (control) (Duncan's new multiple range test).

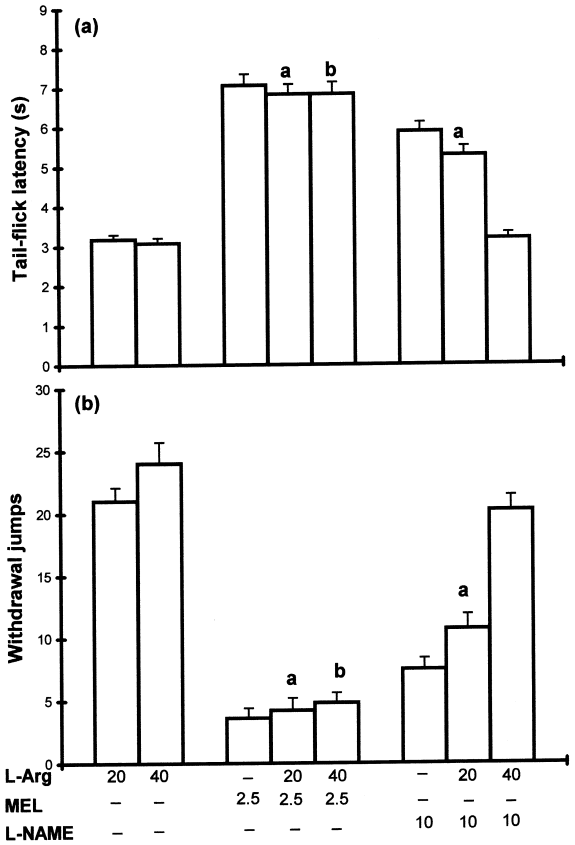

Fig. 7. Effect of chronic administration of L-arginine (L-Arg) and L-NAME, or L-arginine and melatonin on the induction of morphine tolerance (a) and dependence (b) in mice. Along with morphine (10 mg/kg), animals were chronically treated with L-arginine (20 and 40 mg/kg), melatonin (2.5 mg/kg) or L-NAME (10 mg/kg) alone, or combination of L-arginine (20 and 40 mg/kg) and melatonin (2.5 mg/kg), or L-arginine (20 and 40 mg/kg) and L-NAME (10 mg/kg) during the induction phase (days 1–9). These animals received only morphine on day 10 of testing. After the analgesic effect of morphine was recorded, animals were treated with naloxone (2 mg/kg, i.p.) and withdrawal jumping was recorded for the next 20 min. Each bar represents the mean ± S.E.M. (n = 6–8). [a] $P < 0.05$ vs. L-arginine (20 mg/kg) and [b] $P < 0.05$ vs. L-arginine (40 mg/kg) control group (Duncan's new multiple range test).

reversing morphine tolerance in the presence of L-arginine (20 mg/kg). However, unlike the effect of L-NAME, the effect of melatonin was not sensitive to reversal by increasing the dose of L-arginine. Mice treated with L-arginine (20 mg/kg) and L-NAME (10 mg/kg) during the induction phase showed a significant analgesic action to morphine as compared to mice treated with L-arginine (20 mg/kg) alone. On increasing the dose of L-arginine to 40 mg/kg, the effect of L-NAME (10 mg/kg) on the induction of morphine tolerance was completely antagonized. Co-administration of L-arginine (20 mg/kg) with melatonin (2.5 mg/kg) during the induction phase showed did not antagonize the melatonin reversal of morphine tolerance. Also, increasing the dose of L-arginine to 40 mg/kg had no effect on the melatonin reversal of morphine tolerance (Fig. 7a). Although concurrent administration of

L-arginine (40 mg/kg) with morphine during induction phase led to no significant changes in naloxone-precipitated withdrawal jumping, its co-administration with L-NAME (10 mg/kg) during the induction phase completely antagonized the L-NAME-induced suppression of physical dependence on morphine. The melatonin (2.5 mg/kg) reversal of the induction of morphine dependence was not antagonized by either doses of L-arginine (20 and 40 mg/kg) (Fig. 7b).

3.7. Effect of co-administration of L-arginine with L-NAME or melatonin on expression of morphine dependence

Acute administration of L-arginine (20 and 40 mg/kg) during the expression phase exacerbated naloxone-precipitated morphine withdrawal jumping. Administration of L-NAME (10 mg/kg) during the expression phase decreased the incidence of naloxone-precipitated morphine withdrawal jumping, and also its administration before L-arginine (20 mg/kg) during the expression phase reversed the facilitatory effect of L-arginine on the expression of morphine dependence. However, when the dose of L-arginine was increased to 40 mg/kg in the presence of L-NAME (10 mg/kg), L-arginine retained its facilitatory effect on the expression of morphine dependence. The reversal of the expression of morphine dependence induced by the acute administration of melatonin (2.5 mg/kg) was not sensitive to reversal by L-arginine (20 and 40 mg/kg) treatment (Fig. 8).

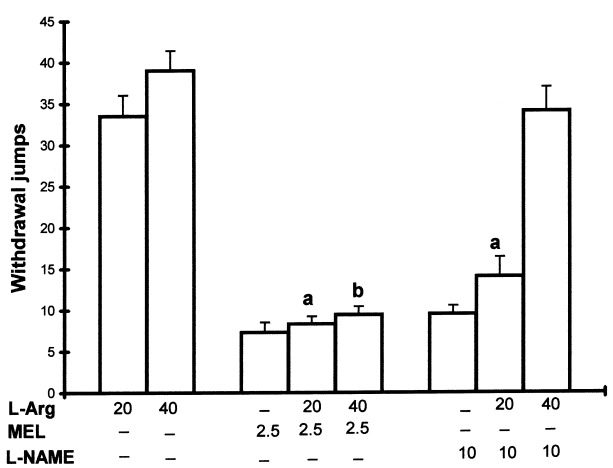

Fig. 8. Effect of acute administration of L-arginine (L-Arg) and L-NAME, or L-arginine and melatonin on the expression of morphine dependence in mice. All the groups of animals received morphine (10 mg/kg, s.c.) alone during the induction period (days 1–9). On the 10th day, along with morphine (10 mg/kg), animals were treated with L-arginine (20 and 40 mg/kg), melatonin (2.5 mg/kg) or L-NAME (10 mg/kg) alone, or its combination with L-arginine (20 and 40 mg/kg) and melatonin (2.5 mg/kg), or L-arginine (20 and 40 mg/kg) and L-NAME (10 mg/kg). After the analgesic effect of morphine was recorded, animals were treated with naloxone (2 mg/kg, i.p.) and withdrawal jumping was recorded for the next 20 min. Each bar represents the mean ± S.E.M. ($n = 6–8$). [a] $P < 0.05$ vs. L-arginine (20 mg/kg) and [b] $P < 0.05$ vs. L-arginine (40 mg/kg) control group (Duncan's new multiple range test).

4. Discussion

The results presented show that co-administration of melatonin during the phase (days 1–9) delayed the development of tolerance to the analgesic effect of morphine and also reversed naloxone-precipitated withdrawal jumping. Acute administration of melatonin during the expression phase (on day 10) reversed only naloxone-induced withdrawal jumping in a dose-dependent manner, but had no effect on tolerance developed to the analgesic effect of morphine. Among the putative melatonin receptor antagonists studied, neither luzindole nor prazosin antagonized the melatonin reversal of morphine tolerance and dependence. The peripheral benzodiazepine receptor antagonist PK11195 but not the central benzodiazepine receptor antagonist flumazenil partially antagonized the melatonin-induced reversal of morphine dependence, but had no effect on morphine tolerance per se. Further studies showed that the action melatonin on the reversal of morphine tolerance and dependence resembled that of L-NAME, a NOS inhibitor. Also melatonin reversed the effect of L-arginine, a NO donor which facilitated morphine tolerance and dependence in a non-competitive manner. These results suggest that the inhibitory effect of melatonin on the development of morphine tolerance and dependence could be due to non-competitive suppression of NOS function.

On the basis of their molecular structure three subtypes of the plasma membrane-bound melatonin receptors have been described, namely MT_1, MT_2 and MT_3 receptor subtypes (Vanecek, 1998). MT_1 and MT_2 receptors are a high-affinity melatonin receptor is coupled to inhibitory G protein (G_i), i.e. they mediate suppression of intracellular cAMP levels. Most of the physiological actions of melatonin are mediated via the high-affinity MT_1 or MT_2 receptor (Vanecek, 1998). Recently, it has been shown that melatonin acting via MT_1 and MT_2 receptor subtypes mediates anxiolytic and analgesic activity (Kopp et al., 1999; Yu et al., 2000), modulates GABA-A receptor function (Wan et al., 1999) and antagonizes 5-HT_{2A} neurotransmitter activity (Eison et al., 1995; Raghavendra and Kulkarni, 2000). The MT_3 receptor is speculated to transduce its signal via increased inositol turnover (Vanecek, 1998). Melatonin increases acetylcholine levels in the nucleus accumbens via MT_3 receptors, as revealed by the reversal of this effect by prazosin, an antagonist for MT_3 receptor (Paredes et al., 1999). It is known that modulators of GABAergic (Ho et al., 1976; Tejawani et al., 1993), cholinergic (Bhargava and Way, 1972; Brase et al., 1974) and 5-HT_{2A} neurotransmission (Gulati and Bhargava, 1988; Neal and Sparber, 1986) affect the development of opioid tolerance and dependence. However, in the present study, the co-administration of luzindole (MT_1 and MT_2 receptor antagonist) or prazosin (MT_3 antagonist) with melatonin failed to antagonize the reversal of morphine tolerance and dependence elicited by melatonin, thereby suggesting that MT_1, MT_2 or MT_3 receptors do not participate in this

action of melatonin. Recently, Yu et al. (2000) reported that melatonin-induced analgesic activity is mediated via MT_2 receptors in rats. In the present study, at the doses selected melatonin failed to induce any analgesic action even after chronic administration. Also, it failed to modify the antinociceptive effect of a submaximal dose of morphine in the tail flick test. The observed discrepancy in our result could be due to the lower dose of melatonin (i.e. 5 mg/kg) selected, whereas Yu et al. (2000) observed the analgesic effect of melatonin at higher doses (i.e. from 30 to 120 mg/kg). However, in this dose range (30–120 mg/kg), the specificity of melatonin receptors in mediating this action is questionable.

In contrast to putative melatonin receptor antagonists, the peripheral benzodiazepine receptor antagonist PK11195, but not the central benzodiazepine receptor antagonist flumazenil, partially antagonized the melatonin reversal of naloxone-precipitated withdrawal jumping in morphine-tolerant mice. However, this drug failed to affect the reversal of morphine tolerance induced by melatonin. This suggests the involvement of peripheral benzodiazepine receptors in the mediation of melatonin-induced reversal of morphine dependence. The involvement of peripheral benzodiazepine receptors in the modulation of morphine tolerance and dependence has been reported earlier from our laboratory (Reddy and Kulkarni, 1997, 1998). The peripheral benzodiazepine receptor agonist 4^1-chlordiazepam (Ro5 4864) dose dependently reversed the development of morphine tolerance and dependence, which was antagonized by PK11195, whereas flumazenil failed to reverse this action. Niles and Hashemi (1990) reported that, like Ro5 4864, melatonin and the non-selective benzodiazepine receptor agonist diazepam inhibited forskolin-stimulated adenylate cyclase activity in various brain regions, an effect which was reversed by PK11195 or pertussis toxin. The central benzodiazepine receptor antagonist flumazenil failed to block the effect of both melatonin and diazepam on adenylate cyclase activity, indicating that this receptor is not involved (Niles and Hashemi, 1990; Tenn et al., 1996). These observations suggest that the melatonin reversal of morphine dependence could be due to its agonistic activity on peripheral benzodiazepine receptors.

It is well known that the second messenger system adenylate cyclase-cAMP plays a role in the actions of opioids (Sharma et al., 1977; Crain and Shen, 1998). cAMP administered during morphine treatment facilitated the development of tolerance and dependence in mice (Ho et al., 1973), and administration of pertussis toxin inhibited naloxone-precipitated withdrawal responses in rats (Paraloro et al., 1990). These observations suggest that pertussis toxin-sensitive G-proteins are necessary for signal transduction in a series of events leading to the induction of morphine tolerance and dependence. Melatonin has been found to decrease the intracellular concentration of cAMP by acting via high-affinity MT_1 and MT_2 receptor subtypes (Vanecek, 1998). However, the failure of luzindole to antagonize the melatonin reversal of morphine tolerance and dependence suggests that these actions are not mediated through putative high-affinity receptors. Consistent with the above observation, Tenn et al. (1996) predicted that inhibition of striatal adenylate cyclase activity by melatonin does not involve the high-affinity G_i protein coupled receptor because micromolar concentrations of melatonin are required to inhibit adenylate cyclase activity in the striatum, whereas pico- and nanomolar concentrations are effective at high-affinity receptors. Moreover, autoradiographic and receptor binding studies in the rat brain have shown that the high-affinity G_i-protein coupled receptor for melatonin is primarily localized in the suprachiasmatic nucleus of the hypothalamus, but not in the striatum. Further, the ability of PK11195 to block the inhibitory effect of melatonin on adenylate cyclase activity provides further evidence that peripheral benzodiazepine receptors, and not high-affinity melatonin receptors are involved in this action (Tenn et al., 1996). Although PK11195 is reported to completely antagonize the 4^1-chlordiazepam induced reversal of morphine tolerance and dependence (Reddy and Kulkarni, 1997, 1998), in the present study PK11195 partially reversed naloxone-precipitated withdrawal jumping but had no effect on the melatonin reversal of morphine tolerance. This suggests that besides the peripheral benzodiazepine receptor, melatonin modulated other neurotransmitter or neuromodulator pathways that have a dominant role in the reversal of morphine tolerance and dependence.

NO plays a key role in the development of morphine tolerance and dependence (Bhargava, 1994; Majeed et al., 1994). The NO level enhanced by chronic morphine may increase the levels of cGMP, leading to phosphorylation of some key proteins and altered physiological responses, finally resulting in morphine tolerance and dependence (Bredt and Snyder, 1989; Lue et al., 1999). Drugs which suppress the synthesis or release of NO are shown to block the development of morphine tolerance and dependence (Bhargava, 1994; Lue et al., 1999). In the present study, chronic administration of the NOS inhibitor L-NAME during the induction phase delayed the development of tolerance to and dependence on morphine, whereas administration of the NO donor L-arginine during the induction phase accelerated the development of tolerance. These observations are in line with the findings of Majeed et al. (1994) and Pataki and Telegdy (1998). Chronic administration of L-arginine during the induction phase led to the early development of complete tolerance, i.e. in the presence of L-arginine, morphine failed to increase the nociceptive threshold of animals after the 6th day, whereas the analgesic effect of morphine alone disappeared after the 9th day. Thus, the early development of complete tolerance in the L-arginine-treated group could explain the failure to observe any significant difference between the nociceptive threshold of the morphine and morphine + L-arginine

treated groups on the 10th day. Also, consistent with the findings of Pataki and Telegdy (1998), chronic administration of L-arginine did not affect morphine dependence. Acute administration of either L-NAME or L-arginine during the expression phase did not alter the nociceptive threshold in morphine-tolerant mice. However, their administration affected the expression of morphine dependence differentially, i.e. L-NAME treatment reversed, whereas L-arginine exacerbated naloxone-precipitated withdrawal jumping. A similar observation was also reported by Adams et al. (1993) in morphine-dependent rats. Recent studies have shown that administration of naloxone to morphine-dependent rats and mice increases neuronal NOS (nNOS) immunoreactivity in various brain regions (Jhamandas et al., 1996; Cuellar et al., 2000). Thus, increased NOS activity leads to the formation of excess NO, which might act as a contributory factor in the neuronal activation that leads to behavioral signs of withdrawal.

In the present study, like the NOS inhibitor L-NAME, melatonin co-administered with L-arginine blocked the facilitation of morphine tolerance induced by L-arginine during the induction phase. Also, prior administration of melatonin or L-NAME during the expression phase reversed the L-arginine-induced facilitation of naloxone-precipitated withdrawal jumping in morphine-tolerant mice. Recently, it has been shown that melatonin inhibits constitutive NOS (cNOS) activity via complex formation with calmodulin, thereby decreasing NO and subsequent elevation of cGMP production in various brain regions (Bettahi et al., 1996; Pozo et al., 1997). It is known that the activation of calmodulin during chronic treatment with morphine is critically involved in the development of morphine tolerance and dependence (Fan et al., 1999). Calmodulin antagonists such as trifluperazine, chlorpromazine and calmidazolium, etc., inhibit some NOS isoenzymes, including cNOS (Forstermann et al., 1991). It is also known that inhibitors of calmodulin-dependent protein kinase or nNOS activity attenuate the development of morphine tolerance and dependence (Bhargava, 1994; Fan et al., 1999). These findings suggest that the melatonin-induced reversal of the development of morphine tolerance and dependence could be due to suppression of Ca^{2+}-calmodulin-dependent NOS activity. However, in the present study, unlike that of L-NAME, the melatonin-induced reversal of tolerance and dependence to morphine was not reversed by increasing the dose of L-arginine. This could be due to non-competitive inhibition of NOS activity by melatonin, i.e. the inhibitory effect of melatonin on NO release is due to inhibition of Ca^{2+}-calmodulin-dependent activation NOS rather than a competitive (with L-arginine) inhibition of NOS function (Pozo et al., 1997).

NO is involved in nociceptive processes, particularly in the spinal cord (Holey et al., 1992). Available reports on the role of NO in the mediation of spinal nociception are controversial. NO donors when given i.t. induced hyperalgesia in rats, whereas NOS inhibitors given i.t. or i.c.v. elicited slight antinociception (Przewlocka et al., 1994). Kabawata et al. (1996) showed that i.c.v. administration of the NO donor L-arginine elicited antinociception in mice, as assessed in the tail-flick test, whereas Dambisya and Lee (1996) and Bhargava et al. (1997) showed that acute or chronic administration of either L-arginine or L-NAME had no effect in the tail-flick test. Our study also did not find any significant change after chronic administration of L-arginine or L-NAME (even melatonin), which is in agreement with the observations of Dambisya and Lee (1996) and Bhargava et al., (1997). Drugs that modify the concentration of NO in the central nervous system also appear to modify opioid-induced antinociception (Brignola et al., 1994). Acute or chronic i.p. or p.o. administration of a very large amount of L-arginine (200 mg/kg and above) reduced morphine antinociception in rats (Przewlocki et al., 1993). In contrast to the above findings, Dambisya and Lee (1996) reported that prolonged inhibition of NO synthesis reduced the antinociceptive response to morphine. These authors showed that chronic administration of lower doses of L-arginine (20 mg/kg, twice daily for 2 days) had no effect on morphine-induced antinociception, whereas chronic administration of L-NAME, but not L-NAAM (10 mg/kg twice daily for 2 days), significantly reversed the acute analgesic effect of morphine. However, in our study chronic administration of either L-NAME (10 mg/kg, twice daily) or L-arginine (15 mg/kg, twice daily) for 9 days failed to modify the acute morphine-induced analgesia in mice. The discrepancy observed between our results and those of previous reports could be due to differences in experimental protocol followed or due to the low dose of L-arginine or L-NAME used in the study. However, in a recent study, Lue et al. (1999) have shown that morphine-stimulated nitrite levels in rat hippocampal slices are maximal at a time when there is no electrophysiological tolerance. This observation suggests that NO mediates some cellular changes to cause the development of morphine tolerance but does not itself have an effect on population spikes. This suggests that modulators of the NO system do not have a role in the mediation of morphine analgesia but may the modulate development of morphine tolerance and dependence by inducing some cellular changes.

Taken together, the present study shows that melatonin reverses the development of tolerance and dependence to morphine. These actions of melatonin are not mediated via central benzodiazepine receptors or through putative melatonin receptors. Further study revealed that the suppression of NOS activity and modulation of peripheral benzodiazepine receptors might have contributed to the mechanism of melatonin-induced reversal of morphine tolerance and dependence.

Acknowledgements

The Senior Research Fellowship (V.R.) of the Council of Scientific and Industrial Research (CSIR), New Delhi,

is gratefully acknowledged. The authors thank Morepen Laboratories, Parwano, India for generously donating melatonin used in this study.

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