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NMDA receptor antagonism disrupts the development of morphine analgesic tolerance in male, but not female C57BL/6J mice

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Submitted 28 November 2005; accepted in final form 27 March 2006

Bryant, Camron D., Shoshana Eitan, Kevin Sinchak, Michael S. Fanselow, and Christopher J. Evans. NMDA receptor antagonism disrupts the development of morphine analgesic tolerance in male, but not female C57BL/6J mice. *Am J Physiol Regul Integr Comp Physiol* 291: R315–R326, 2006. First published April 6, 2006; doi:10.1152/ajpregu.00831.2005.—Multiple studies demonstrate that coadministration of *N*-methyl-D-aspartate (NMDA) receptor antagonists with the opioid agonist morphine attenuates the development of analgesic tolerance. Sex differences in the effects of noncompetitive, but not competitive NMDA receptor antagonists on acute morphine analgesia, have been reported in mice, yet the role of sex in modulation of morphine tolerance by NMDA receptor antagonists has yet to be addressed. Therefore, we tested whether there is a sex difference in the effect of NMDA receptor antagonists on the development of morphine analgesic tolerance in C57BL/6J mice. Acutely, at a dose required to affect morphine tolerance in male mice, the noncompetitive NMDA receptor antagonist dizocilpine (MK-801) prolonged morphine analgesia similarly in both sexes in the hot plate and tail withdrawal assays. In the hot plate assay, coadministration of MK-801 or the competitive antagonist 3-(2-carboxypiperazin-4-yl)propyl-1-phosphanoic acid (CPP) with morphine attenuated the development of tolerance in male mice, while having no effect in females. Like normal and sham females, ovariectomized mice were similarly insensitive to the attenuation of morphine tolerance by MK-801 in the hot plate assay. Surprisingly, in the tail withdrawal assay, MK-801 facilitated the development of morphine-induced hyperalgesia and tolerance in males but not females. The results demonstrate that male mice are more sensitive to modulation of nociception and morphine analgesia after repeated coadministration of NMDA receptor antagonists. Furthermore, the underlying mechanisms are likely to be different from those mediating the sex difference in the modulation of acute morphine analgesia that has previously been reported.

pain; antinociception; opioid; hyperalgesia; ovariectomy

ANALGESIC TOLERANCE TO OPIOID drugs is a clinical problem because progressively higher doses are required for chronic pain relief, which can limit the safety and exacerbate withdrawal symptoms. In addition to opioid receptor-level cellular adaptations associated with tolerance (reviewed in Ref. 5), a separate focus of tolerance mechanisms highlights the impor-

tance of systems adaptations that oppose the acute effects of opioids (e.g., glutamate, substance P, cholecystokinin, and dynorphin) and contribute to opponent behavioral and psychological responses associated with tolerance and dependence (e.g., hyperalgesia and dysphoria; recently reviewed in Ref. 11). Adaptations, such as hyperalgesia can have an associative learning component, which contributes to tolerance (58).

Many studies have shown that coadministration of noncompetitive or competitive NMDA receptor antagonists can attenuate the development of analgesic tolerance to morphine in rodents (62), but the mechanism of this effect remains unclear. It is believed that NMDA receptor antagonists block calcium-mediated signaling and the resulting changes in gene expression and neuroplasticity that mediate morphine tolerance.

In considering the mechanisms by which NMDA receptor antagonists disrupt morphine tolerance, it may be useful to consider their effects on acute morphine analgesia. Although some studies indicate that NMDA receptor antagonists potentiate or prolong morphine analgesia, others show no difference or just the opposite. Discrepancies may be ultimately explained by many different factors, including the type and dose of antagonist (16), morphine dose, sex (51), species, strain, route of drug administration, injection-to-test interval, antagonist-to-morphine interval, and type of nociceptive assay, to name a few.

It was recently reported that noncompetitive NMDA receptor antagonists [dizocilpine (MK-801) and dextromethorphan] enhanced acute morphine analgesia in male but not female mice (25, 51). The lack of enhancement by dextromethorphan in females was estrogen-dependent as ovariectomized (ovx) mice showed a male-like enhancement and estrogen replacement reinstated the female phenotype (25). In contrast, the competitive NMDA receptor antagonist LY235959 enhanced morphine analgesia equally in both sexes (51). In rats, sex differences in the modulation of morphine analgesia by MK-801 have also been reported, with one report indicating that males were more sensitive (16) and another report demonstrating that females were more sensitive (29). The discrepancy

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may be due to an interaction of different doses of MK-801 and morphine.

It is unclear whether the effects of NMDA receptor antagonists on acute morphine analgesia are related to their ability to affect morphine tolerance. Simonnet and colleagues (13, 40, 59) have proposed that the enhancement of acute morphine analgesia by NMDA receptor antagonists is due to a blockade of an opponent hyperalgesia (referred to as acute tolerance) that is unmasked by an opioid receptor antagonist after the first opioid exposure. From this view, opioid tolerance has been conceptualized as a pain "sensitization" process, whereby repeated intermittent administration of opioids results in a progressive increase in the activation of pain facilitatory systems (13, 14). Thus, if repeated hyperalgesia induces the changes in gene expression and neuroplasticity that mediates this sensitization process associated with tolerance and if the male-specific enhancement of acute morphine analgesia in mice is actually a male-specific blockade of acute hyperalgesia, then chronic coadministration of the noncompetitive NMDA receptor antagonist MK-801 with morphine should attenuate the development of morphine tolerance in male, but not female mice. In contrast, because qualitatively similar effects were observed previously with competitive NMDA receptor antagonists on acute morphine analgesia, the prediction is that coadministration of the competitive NMDA receptor antagonist 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) with morphine would attenuate the development of morphine tolerance in both male and female mice.

In pilot experiments, we first determined doses of MK-801 and CPP that were required to attenuate morphine tolerance in male mice. We then used these doses to examine the modulation of morphine tolerance in male and female mice. Upon observing a sex difference in the modulation of tolerance in the hot plate and tail withdrawal assays, we then determine whether, under those conditions, there was a sex difference in the modulation of acute morphine analgesia by MK-801. For clarity, in each experiment, the acute data are presented first followed by the tolerance data.

MATERIALS AND METHODS

Animals and surgery. Male and female C57BL/6J mice were obtained from either Jackson Laboratories, Bar Harbor, ME (hot plate; JAX) or from a breeding facility on the University of California at Los Angeles campus (tail withdrawal) and were 8- to 12-wk old at the start of the experiments. Ovariectomies were performed at 8 wk of age in JAX mice, as previously described (48). Briefly, mice were anesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg ip, respectively), and the skin and abdominal wall overlying the ovary were incised. The ovary and uterine horn were exposed. The uterine horn was clamped with a hemostat proximal to the ovary and ligated with dissolvable suture. The ovary was cut from the uterine horn and removed, and the uterine horn was replaced in the body cavity. The abdominal wall was sutured with sterile 5-0 gut and the overlying skin was closed with wound clips. Sham mice were anesthetized, and the skin and abdominal wall overlying the ovary were incised. The abdominal wall was sutured with sterile 5-0 gut, and the overlying skin was closed with wound clips. Mice were allowed at least 1 mo to recover before testing. Although no biochemical assay was conducted to measure estrogen levels, ovx mice weighed significantly more than shams [$t(83) = -4.82$; $P < 0.0001$], and their hair grew back much faster than sham mice after surgery. Both of these observations are consistent with elimination of estrogen function (3, 15, 24, 57). All

mice were housed in a climate-controlled vivarium on a 12:12-h light-dark cycle and had unlimited access to food and water. Treatment and testing were always conducted during the light part of the cycle. Experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee. Separate animals were always used for each pain assay, acute experiment, tolerance experiment, and treatment.

Drugs. Morphine sulfate was obtained from the National Institute on Drug Abuse (Bethesda, MD). The noncompetitive NMDA receptor antagonist MK-801 and the competitive NMDA receptor antagonist CPP were purchased from Sigma (St. Louis, MO). Limited reports investigating the competitive NMDA receptor antagonist D-CPPene (the active analog of \pm CPP) suggest that, similar to other competitive antagonists, this drug potentiates (7), or at least, prolongs morphine analgesia (9) in males. All drugs were dissolved in physiological saline (0.9% NaCl) and administered in a volume of 10 ml/kg.

Pain assays. We used two different pain assays to measure morphine analgesia. First, we used the 52.5°C hot plate assay (20), in which the mice were placed on a hot metal surface and the latency to lick the hindpaw was recorded to the nearest 0.1 s using a stopwatch. A cutoff of 60 s was used to prevent tissue damage.

To test the generality of the hot plate findings to another pain assay, we also used the 49.0°C tail withdrawal/immersion assay (8). Here, the mice were momentarily placed in a restraint made of cotton, and the distal half of their tails were immediately placed into a circulating water bath that was accurate to the nearest 0.1°C. The latency to flick the tail was recorded with a stopwatch to the nearest 0.1 s. A cutoff of 15 s was used to prevent tissue damage. The experimenter was always blind on test day to pretreatment conditions.

Acute morphine analgesia. To test for MK-801 modulation of acute morphine analgesia and tolerance, different doses of MK-801 were used in the hot plate and tail withdrawal assays. This is because the drug regimen using a constant dose of morphine and MK-801 (10 mg/kg and 0.25 mg/kg, respectively) did not produce tolerance in the tail withdrawal assay. Using a second regimen of escalating doses of morphine to induce tolerance in both assays, we found that a higher dose of MK-801 (1 mg/kg) was required to affect tolerance, and thus this dose was examined in the tail withdrawal assay.

In examining acute morphine analgesia in the hot plate assay, mice were measured for baseline latencies once per day for 3 days before analgesic assessment. This was done to eliminate any learning effect on the hot plate latencies to maximize the possibility for detecting a true MK-801-induced hyperalgesia (1). No significant differences were observed between *days 2* and *3*, so these values were averaged and presented as the baseline latency (0 min postinjection; Fig. 1, *A* and *B*). Thirty minutes after the last baseline measurement, mice were administered MK-801 (0.25 mg/kg ip) after 30 min later by morphine (7.5 mg/kg sc; MK+mor). Mice receiving morphine alone were administered an intraperitoneal injection of saline followed 30 min later by morphine (7.5 mg/kg sc; mor). Control mice were given two saline injections (ip and sc), separated by 30 min. Mice receiving MK-801 alone (MK) were administered MK-801 (0.25 mg/kg ip) followed 30 min later by a subcutaneous saline injection. Postinjection latencies were determined every 30 min for 90 min.

In examining the effect of MK-801 on acute morphine analgesia in the tail withdrawal assay, two separate baseline measurements were recorded, separated by 30 min. No significant differences were observed; thus, they were averaged and presented as the baseline (0 min postinjection; Fig. 4, *A* and *B*). Thirty minutes later, mice were administered MK-801 (1 mg/kg ip) followed immediately by morphine (7.5 mg/kg sc; MK+mor). Mice receiving morphine alone were administered an intraperitoneal injection of saline followed immediately by morphine (7.5 mg/kg sc; mor). Control mice were administered two saline injections (ip and sc). Mice receiving MK-801 alone were administered MK-801 (1 mg/kg ip) followed immediately by a subcutaneous saline injection. Postinjection latencies were measured

from 30 to 90 min. Examination of the interaction of MK-801 with higher doses of morphine (20–40 mg/kg) was not possible because all mice reached cutoff latency.

Morphine analgesic tolerance in the hot plate assay using a constant dose of morphine. In the hot plate assay, naïve mice were treated twice daily for 6 days with MK-801 (0.25 mg/kg ip) followed 30 min later by morphine (10 mg/kg sc; MK+mor) (26). The other groups were treated as described for the acute experiment. Injections were conducted at 900 and 1800. On day 7 at 0900, mice were tested for baseline hot plate latencies, and 30 min later, were given a challenge dose of morphine (10 mg/kg sc). Postinjection latencies were measured from 30 to 90 min. Tolerance was indicated by a change in morphine analgesic efficacy after a challenge dose, as previously employed by Hamdy et al. (26).

Morphine analgesic tolerance in the hot plate and tail withdrawal assays using escalating doses of morphine. A second tolerance regimen adapted from a previously published protocol (21) was also examined. This was done to eliminate the possibility that the hot plate results were due to specific parameters of drug administration and because tolerance did not develop in the tail withdrawal assay under the regimen of a constant dose of morphine. Mice were administered escalating doses of morphine (10–40 mg/kg sc) once per day for 6 days. MK-801 (1 mg/kg ip) was coadministered immediately after morphine and then 2 h later (1 mg/kg ip; MK+mor). CPP was coadministered simultaneously with morphine (30 mg/kg ip; CPP+mor) and then 2 h later (30 mg/kg ip). The other groups were treated as described for the acute experiment except for the extra injection of saline (ip; control, mor) or antagonist (ip; MK, CPP) 2 h later. The doses of antagonists for this second tolerance regimen were chosen based on pilot hot plate data using males where lower doses of antagonists with morphine were completely ineffective in attenuating the development of morphine tolerance. Additionally, a second injection of antagonist was necessary to attenuate morphine tolerance, and we chose 2 h based on a previous study, indicating that the half-life of MK-801 is ~2 h (64). Injections were conducted at 1000 and 1200. On day 7 at 1000 h, baseline latencies were first recorded with the hot plate or tail withdrawal assay. Thirty minutes later, all mice received either 10 mg/kg sc morphine (hot plate) or 7.5 mg/kg sc morphine (tail withdrawal). Postinjection latencies were recorded every 30 min from 30 to 90 min and are presented as the percent maximum possible effect as defined in the analysis. As with the regimen of a constant dose of morphine, morphine tolerance was indicated by a change in analgesic efficacy after a single challenge dose. Because this regimen of escalating doses produced a more prolonged tolerance and attenuation of tolerance by MK-801 in males in the hot plate assay, this regimen was used in the ovariectomy experiment (Fig. 3).

Analysis. In the acute experiments, two-way repeated-measures ANOVA of postinjection latencies from 30 to 90 min followed by planned comparison was used for analysis. Paired Student's *t*-test was used in the case of MK-801-induced hyperalgesia that was observed in the tail withdrawal assay. In the tolerance experiments, because of the changes in baseline latencies after repeated administration, postinjection latency values were converted to percent maximum possible effect (%MPE) for each individual animal with respect to each individual baseline value according to the following formula: %MPE = (postinjection latency – baseline latency)/(60 – baseline latency) × 100. Because there was no interaction over time, %MPE values were averaged from 30 to 90 min and two-way ANOVA followed by planned comparison was used. The only exception was with Fig. 1, C and D, in which there was a three-way interaction of time, sex, and treatment. In this instance, two-way repeated-measures ANOVA followed by planned comparison was used for analysis. For the tail withdrawal, because tolerance was only significant at 30 min, this was the time point used in analysis of the %MPE data. For all experiments involving repeated drug administration, area under the curve (AUC; min × %MPE) was estimated from 0 to 90 min using the

trapezoid method. In all analyses, an alpha level of 0.05 was considered significant.

RESULTS

In the hot plate assay, MK-801 prolongs acute morphine analgesia in male and female mice. We observed a similar prolongation of acute morphine analgesia by MK-801 in both sexes in the hot plate assay (Fig. 1, A and B). There was a main effect of treatment [$F(3,47) = 74.7$; $P < 0.0001$], time [$F(2,94) = 7.4$; $P = 0.001$], and an interaction of treatment with time [$F(6,94) = 8.0$; $P < 0.001$]. In comparing mice receiving morphine alone (mor) with mice receiving MK-801 plus morphine (MK+mor), coadministration prolonged morphine analgesia at 90 min in both males [$F(6,94) = 5.0$; $P < 0.05$] and females [$F(6,94) = 4.9$; $P < 0.05$]. There was no change in baseline latency after saline (control) or MK-801 alone (MK) over 90 min in either sex, nor did MK-801 itself have any effect on initial baseline latencies within each animal (Fig. 1, A and B).

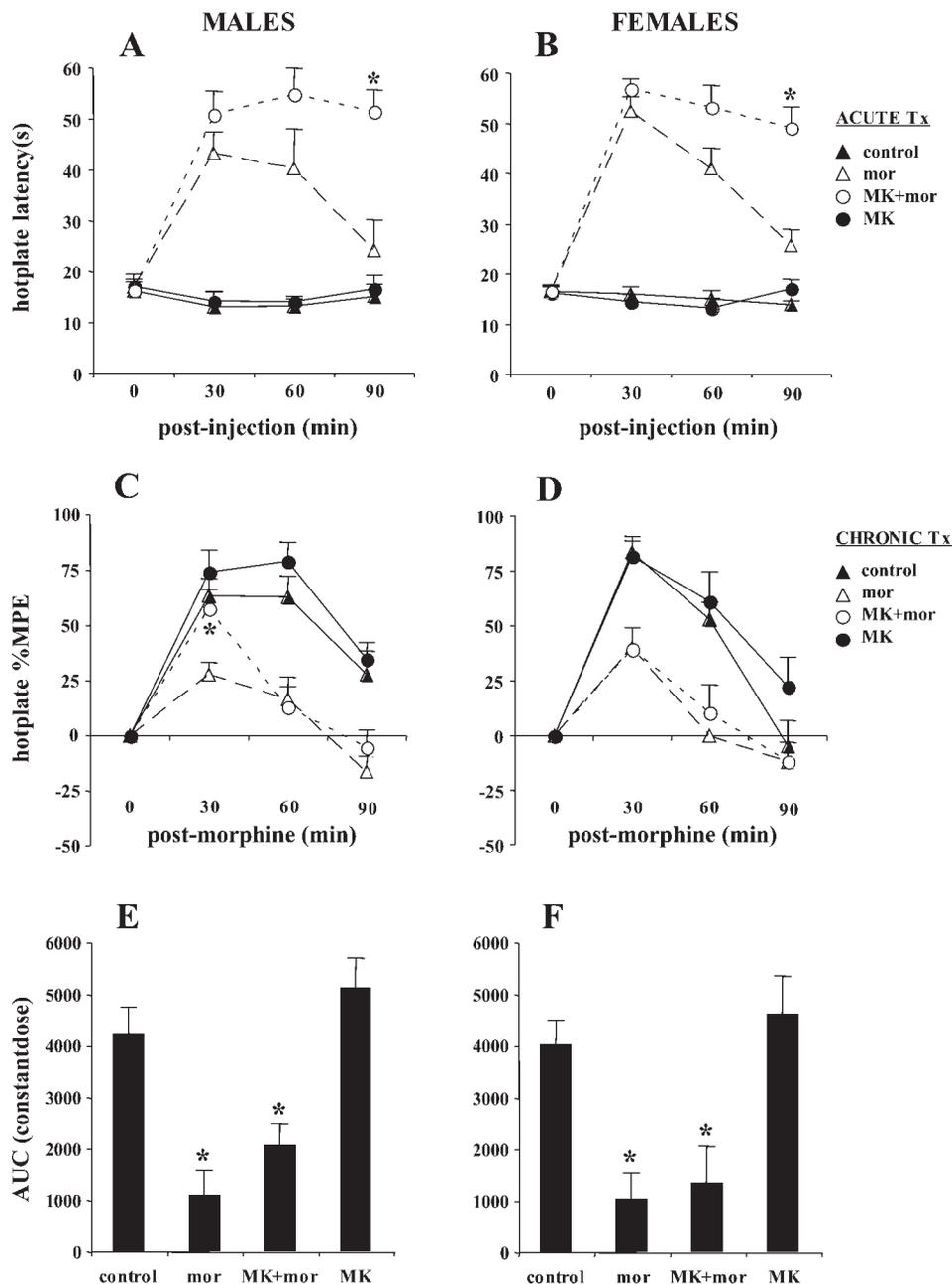
In the hot plate assay, after a constant dose of morphine, MK-801 attenuates the development of morphine tolerance in male, but not female, mice. In naïve mice, after the regimen of repeated administration of constant doses of morphine and MK-801, cotreatment produced a subsequent male-specific increase in baseline latency (herein referred to as baseline hypoalgesia) (Table 1), which was accompanied by a male-specific attenuation of the development of morphine tolerance (Fig. 1C).

In Table 1, in examining changes in baseline latencies after repeated administration, Fisher's paired least significant difference (PLSD) test indicated that male, but not female, mice receiving repeated MK+mor demonstrated significant baseline hypoalgesia relative to control mice ($P < 0.05$).

In Fig. 1, C and D, in examining modulation of morphine tolerance after repeated administration and a subsequent morphine challenge, there was a main effect of treatment [$F(3,80) = 20.7$; $P < 0.0001$] and a treatment by sex by time interaction [$F(6,160) = 2.3$; $P < 0.05$]. In comparing mor vs. control groups, significant tolerance developed at 30, 60, and 90 min in males [$F(6,160) = 5.6$; $P < 0.05$; $F(3,80) = 9.2$; $P < 0.01$; $F(3,80) = 8.4$; $P < 0.01$], whereas in females, tolerance was significant only at 30 and 60 min [$F(6,160) = 6.9$; $P < 0.05$, $F(3,80) = 10.2$; $P < 0.01$]. In males, in comparing mor and MK+mor groups, the development of morphine tolerance was attenuated with MK-801 only at 30 min [$F(6,160) = 3.9$; $P = 0.05$]. In females, there was no effect of MK-801 on the development of morphine tolerance (Fig. 1, C and D).

In Fig. 1, E and F, in examining AUC, there was a main effect of treatment [$F(3,80) = 21.7$; $P < 0.0001$]. In comparing mor and control mice, significant tolerance developed in both male [$F(3,80) = 17.0$; $P < 0.0001$] and female mice [$F(3,80) = 13.2$; $P < 0.001$]. In comparing MK+mor with control mice, tolerance also developed in males [$F(3,80) = 8.1$; $P < 0.01$] and females [$F(3,80) = 10.6$; $P < 0.01$]. In comparing mor mice with MK+mor mice, coadministration of MK-801 with morphine had no effect on the development of tolerance in male or female mice (Fig. 1, E and F).

Fig. 1. *A* and *B*: in the hot plate assay, dizocilpine (MK-801) prolongs acute morphine analgesia in male and female mice. After measurement of baseline hot plate latencies, male and female mice ($n = 5-10$) were administered MK-801 (0.25 mg/kg ip) followed by morphine 30 min later (7.5 mg/kg sc). Postinjection latencies were recorded every 30 min for 90 min. Compared with mice that received MK-801 plus morphine (MK+mor) with those receiving acute morphine (mor), there was a significant prolongation of acute morphine analgesia by MK-801 in male and female mice at 90 min (*). Data are expressed as mean percent hot plate latency \pm SE. *C* and *D*: in the hot plate assay, after a constant dose of morphine, MK-801 attenuates the development of morphine tolerance in male, but not female mice. Male and female mice ($n = 10-12$) were administered MK-801 (0.25 mg/kg ip) followed by morphine 30 min later (10 mg/kg sc) twice daily for 6 days. On *day 7*, after baseline measurements, mice were administered a challenge dose of morphine (10 mg/kg sc) and tested for analgesia every 30 min for 90 min. Significant tolerance developed in males and females at 30 min post-morphine in comparing mice receiving repeated morphine (mor) with those receiving repeated saline (control). Furthermore, in comparing mice receiving repeated MK-801 plus morphine (MK+mor) with those receiving repeated mor, a significant attenuation of the development of tolerance was observed in males (*), but not in females. Data are presented as the mean percent maximum possible effect (%MPE) \pm SE. *E* and *F*: estimations of the area under the curve (AUC) are presented for the tolerance experiment (*C* and *D*) as the mean \pm SE. In considering the entire time-effect curve, there was no significant effect of MK-801 on the development of morphine tolerance in either sex, as both mice receiving repeated mor and those receiving repeated MK+mor showed significantly less analgesia than mice receiving repeated saline (control). Two-way ANOVA (treatment and sex) followed by planned comparison was used for analysis. A *P* value of 0.05 was considered significant.



In the hot plate assay, after escalating doses of morphine, MK-801, and CPP attenuate the development of morphine analgesic tolerance in male, but not female, mice. Using separate naïve mice, repeated coadministration of MK-801 or CPP with escalating doses attenuated the development of morphine analgesic tolerance in male, but not female, mice (Fig. 2). With respect to MK-801, this was accompanied by a male-specific baseline hypoalgesia after coadministration (Table 2).

In Table 2, in examining differences in baseline latencies after repeated administration in the MK-801 experiment, there was a main effect of treatment [$F(3,91) = 7.3$; $P < 0.001$] and sex [$F(1,91) = 7.3$; $P < 0.01$]. Male mice receiving MK+mor and those receiving just MK-801 exhibited significantly higher baseline latencies than control mice on test day [$F(3,91) = 7.2$;

Table 1. Changes in baseline latencies in the hot plate assay after a regimen of a constant dose of morphine with MK-801

Sex	Chronic Tx	Hot Plate
male	saline	17.8 \pm 1.8
male	morphine	21.1 \pm 1.2
male	MK + mor	22.5 \pm 1.3*
male	MK-801	19.1 \pm 1.1
female	saline	19.6 \pm 2.1
female	morphine	20.1 \pm 1.8
female	MK + mor	20.8 \pm 1.7
female	MK-801	22.2 \pm 2.3

Data are presented as the mean baseline latency \pm SE. Mice were treated twice daily for 6 days with dizocilpine (MK-801) (0.25 mg/kg ip) followed 30 min later by morphine (10 mg/kg sc). On *day 7*, baseline hot plate latencies were measured. *Significantly different from control mice receiving chronic saline. An alpha level of 0.05 was considered significant. MK + mor, MK-801 + morphine.

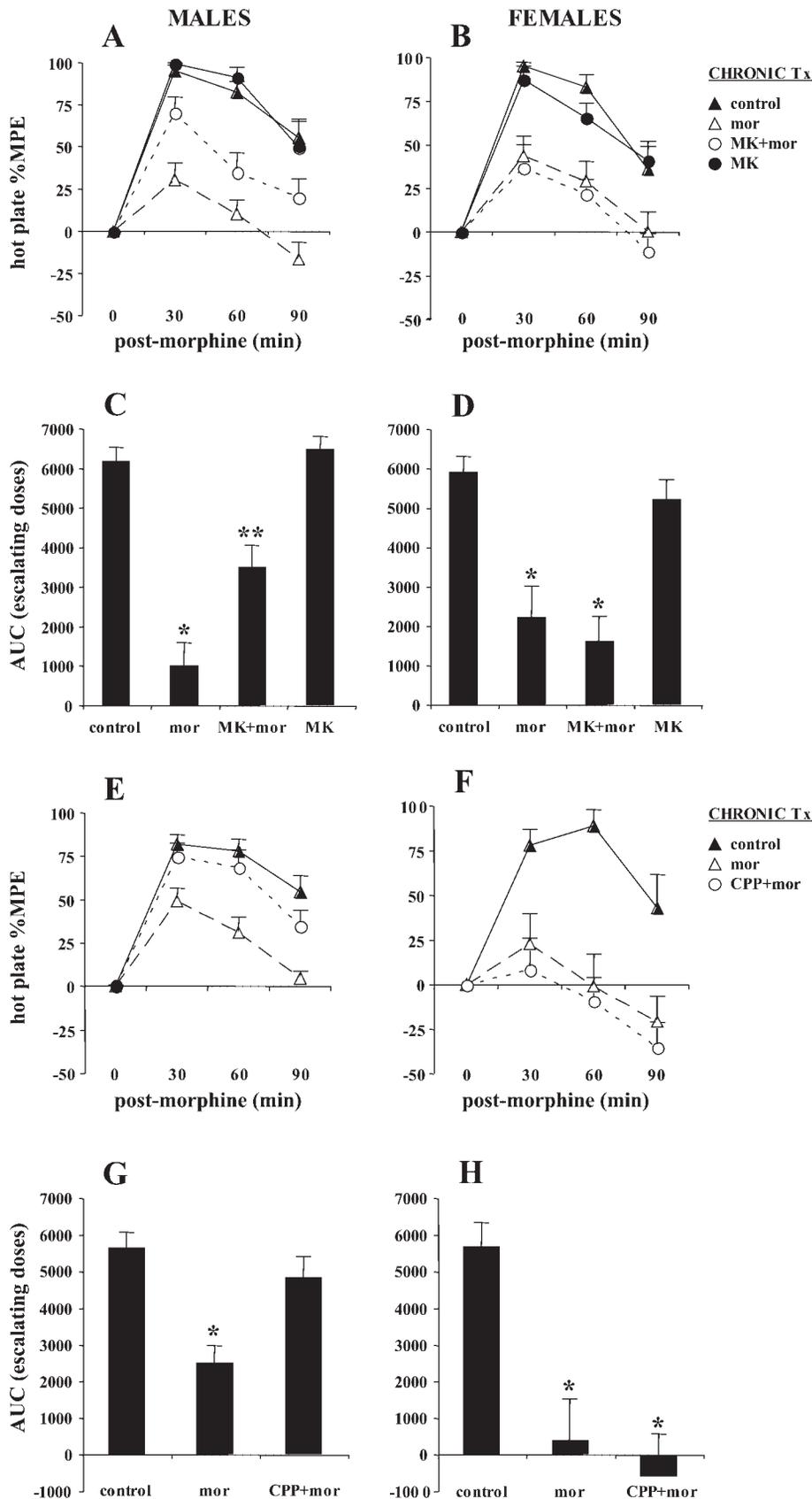


Fig. 2. In the hot plate assay, after escalating doses of morphine, coadministration of MK-801 or 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) attenuates the development of morphine tolerance in male, but not in female mice. *A* and *B*: mice ($n = 10-14$) were coadministered the noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 (1 mg/kg ip) and morphine (10–40 mg/kg sc) once per day for 6 days. On *day 7*, baseline latencies were measured, mice were then injected with morphine (10 mg/kg sc), and postinjection latencies were recorded every 30 min for 90 min. Significant tolerance developed in both males and females when comparing mice receiving mor with those receiving repeated saline (control). However, MK-801 and CPP attenuated the development of morphine tolerance in male, but not female, mice as male mice repeatedly coadministered MK-801 or CPP with morphine (MK+mor; CPP+mor) exhibited significantly greater analgesia on test day than mice receiving mor. Data are expressed as mean %MPE \pm SE. *C* and *D*: estimations of the AUC are presented for the tolerance experiment involving MK-801 (*A* and *B*) as the means \pm SE. Male, but not female, mice repeatedly administered MK+mor show significantly greater analgesia than mice receiving mor, indicating a male-specific attenuation of morphine tolerance (**). *Significantly different from control mice. *E* and *F*: mice ($n = 7-14$) were coadministered the competitive NMDA receptor antagonist CPP (30 mg/kg ip) and morphine (10–40 mg/kg sc), once per day for 6 days. On *day 7*, baseline latencies were measured; mice were then injected with morphine (10 mg/kg sc), and postinjection latencies were recorded every 30 min for 90 min. A similar male-specific attenuation of tolerance was also observed after repeated coadministration of the competitive NMDA receptor antagonist CPP with morphine. Data are expressed as mean %MPE \pm SE. *G* and *H*: estimations of the AUC are presented for the tolerance experiment involving CPP (*E* and *F*) as the means \pm SE. Male, but not female, mice repeatedly administered CPP plus morphine (CPP+mor) show significantly greater analgesia than mice receiving mor, indicating a male-specific attenuation of morphine tolerance (*). For all experiments, two-way ANOVA (treatment and sex) followed by planned comparison was used for analysis (see MATERIALS AND METHODS). A *P* value of 0.05 was considered significant.

Table 2. Changes in baseline latencies after a regimen of escalating doses of morphine with MK-801

Sex/Sx	Chronic Tx	Hot Plate	Tail Withdrawal
male	saline	19.2±1.2	4.2±0.2
male	morphine	23.3±2.1	3.3±0.3*
male	MK + mor	27.3±3.1*	2.6±0.2**
male	MK-801	31.3±2.5*	3.8±0.3
female	saline	17.3±1.6	3.4±0.3
female	morphine	18.7±2.0	2.4±0.2*
female	MK + mor	22.1±2.4	2.2±0.2*
female	MK-801	25.4±2.9*	3.2±0.2
sham	saline	18.9±1.6	ND
sham	morphine	20.6±1.6	ND
sham	MK + mor	21.4±1.4	ND
ovx	saline	15.9±1.1	ND
ovx	morphine	17.9±1.3	ND
ovx	MK + mor	22.6±1.3*	ND

Data are presented as mean baseline latency ± SE. Mice were treated once daily for 6 days with MK-801 (1 mg/kg ip) and escalating doses of morphine (10–40 mg/kg sc). On day 7, baseline hot plate or tail withdrawal latencies were measured. *Significantly different from control mice receiving chronic saline. **Significantly different from mice receiving repeated morphine. An alpha level of 0.05 was considered significant. Sx, surgery; Tx, treatment; ovx, ovariectomized; ND, not determined.

$P < 0.01$; $F(3,91) = 14.7$; $P < 0.001$, respectively]. In contrast, only female mice receiving repeated MK exhibited baseline hypoalgesia relative to control mice receiving repeated saline [$F(3,91) = 5.9$; $P < 0.05$]. In examining possible changes in baseline latencies after repeated coadministration of CPP with morphine, there was no effect on either sex (Table 2).

In Fig. 2, A and B, in examining the modulation of morphine tolerance by MK-801, there was a main effect of treatment [$F(3,91) = 31.8$; $P < 0.0001$] and an interaction of treatment and sex [$F(1,91) = 2.90$; $P < 0.05$]. In comparing control and mor mice, significant tolerance developed in both males [$F(3,91) = 48.8$; $P < 0.0001$] and females [$F(3,91) = 19.04$; $P < 0.0001$]. However, in comparing mor mice with MK+mor mice, coadministration of MK-801 attenuated the development of tolerance in males [$F(3,91) = 11.5$; $P < 0.01$] but not females (Fig. 2, A and B).

In Fig. 2, C and D, in examining AUC, there was a main effect of treatment [$F(3,91) = 36.2$; $P < 0.0001$] and an interaction of treatment with sex [$F(3,91) = 3.2$; $P < 0.05$]. In comparing mor mice with control mice, significant tolerance developed in both males [$F(3,91) = 53.4$; $P < 0.0001$] and females [$F(3,91) = 23.2$; $P < 0.0001$]. In comparing mor with MK+mor mice, coadministration of MK-801 with morphine attenuated the development of tolerance in males [$F(3,91) = 12.3$; $P < 0.001$], but not females. The attenuation in males was incomplete as MK+mor mice still exhibited significant tolerance relative to control [$F(3,91) = 14.4$; $P < 0.001$]. Female mice receiving MK+mor exhibited tolerance relative to control mice [$F(3,91) = 28.5$; $P < 0.0001$] that was similar to mice receiving mor (Fig. 2, C and D).

In male mice, a lower dose of MK-801 (0.3 mg/kg ip) was completely ineffective in attenuating the development of morphine tolerance under the regimen of escalating doses of morphine (data not shown). Specifically, in examining AUC, there was a main effect of treatment [$F(3,24) = 15.7$; $P < 0.0001$]. Compared with mice receiving repeated saline (control), Fisher's PLSD indicated that significant tolerance devel-

oped in mice receiving repeated mor ($P < 0.001$) and in mice receiving repeated MK+mor; ($P < 0.001$) and that mor mice and MK+mor mice did not differ from each other ($P > 0.05$).

In Fig. 2, E and F, in the CPP experiment, after repeated administration and a subsequent morphine challenge, there was a main effect of treatment [$F(2,61) = 21.2$; $P < 0.0001$], sex [$F(1,61) = 19.7$; $P < 0.0001$], and an interaction of treatment with sex [$F(2,61) = 7.2$; $P < 0.01$]. In comparing control and mor mice, tolerance developed in both males [$F(2,61) = 17.5$; $P < 0.0001$] and females [$F(2,61) = 20.5$; $P < 0.0001$]. In males, the development of tolerance was attenuated by coadministration of CPP with morphine as CPP+mor mice differed significantly from mor mice [$F(2,61) = 9.0$; $P < 0.01$]. In contrast, in females, there was no effect of coadministration on tolerance, as CPP+mor mice differed from control mice [$F(2,61) = 28.5$; $P < 0.0001$], but not mor mice (Fig. 2, E and F).

In Fig. 2, G and H, in examining AUC, there was a main effect of treatment [$F(2,61) = 20.7$; $P < 0.0001$], sex [$F(1,61) = 18.9$; $P < 0.0001$], and an interaction of treatment with sex [$F(2,61) = 7.5$; $P < 0.01$]. In comparing mor mice with control mice, significant tolerance developed in both males [$F(2,61) = 16.1$; $P < 0.001$] and females [$F(2,61) = 20.8$; $P < 0.0001$]. In comparing mor with CPP+mor mice, coadministration of CPP with morphine completely blocked the development of morphine tolerance in males, as these groups differed significantly from each other [$F(2,61) = 9.0$; $P < 0.01$], and CPP+mor mice did not differ from control mice. In females, coadministration had no effect on the development of morphine tolerance as CPP+mor mice differed from control mice [$F(2,61) = 28.5$; $P < 0.0001$], but not from mor mice (Fig. 2, G and H).

We did not run a group receiving repeated CPP alone. This does not in any way limit the interpretation of the data because importantly, there was no change in baseline latency after repeated CPP+mor relative to control mice and in a separate experiment, we found no effect of repeated administration of CPP alone on baseline latencies in either males [$t(12) < 1$] or females [$t(12) < 1$] (data not shown).

Ovariectomy has no effect on the lack of attenuation of the development of morphine analgesic tolerance by MK-801. Because morphine tolerance and the male-specific attenuation of tolerance with MK-801 in the hot plate assay were both more prolonged after the second regimen, using escalating doses of morphine, we used this regimen for examining the effect of MK-801 on the development of morphine tolerance in sham and ovx mice. To minimize the number of mice used, the group receiving repeated MK-801 alone was excluded because we previously demonstrated that there was no effect of repeated MK-801 on subsequent acute morphine analgesia in either sex (Fig. 2, A and B). The results indicate that after repeated coadministration of MK-801 plus morphine, ovx reinstated the male-like baseline hypoalgesia (Table 2). However, like sham mice, ovx mice were insensitive to modulation of the development of morphine tolerance after repeated administration of MK-801 with morphine (Fig. 3).

In Table 2, in examining possible changes in baseline latencies after repeated administration, there was a main effect of treatment [$F(2,79) = 5.0$; $P < 0.01$]. Significant baseline hypoalgesia developed in ovx mice treated with MK-801 plus morphine [$F(2,79) = 9.3$; $P < 0.01$] (Table 2).

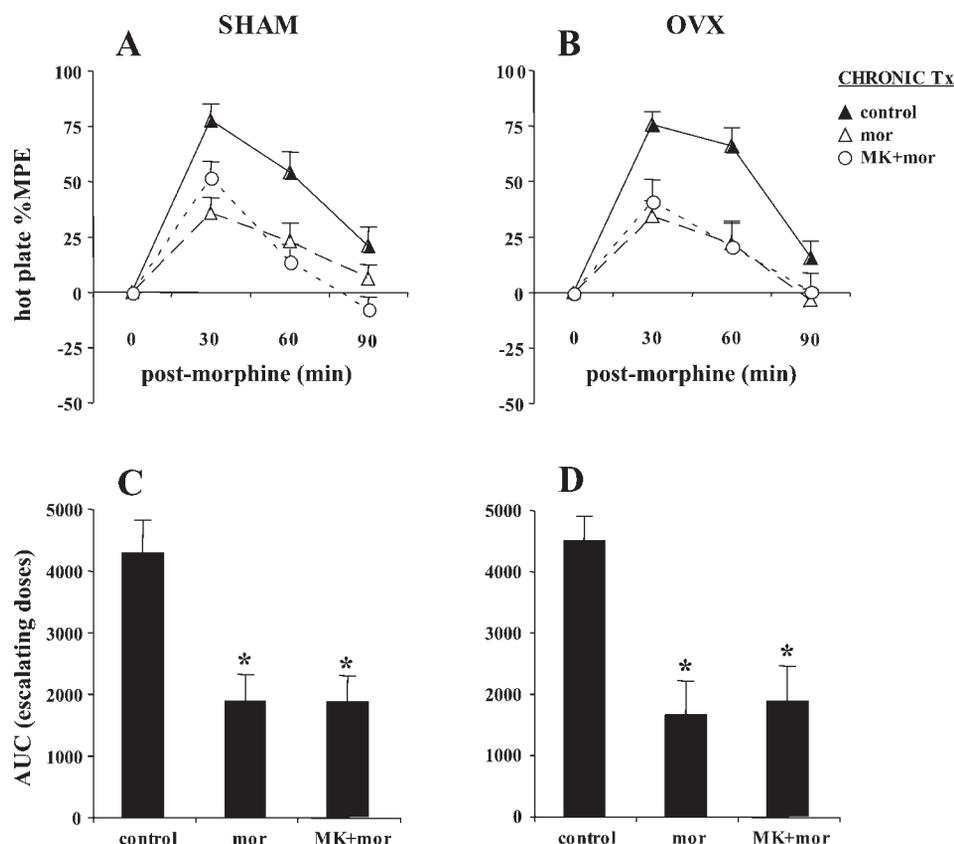


Fig. 3. Ovariectomy has no effect on the lack of attenuation of the development of morphine tolerance by MK-801. A and B: sham ($n = 15-17$) and ovariectomized (ovx) ($n = 11-13$) mice were treated for 6 days with MK-801 (1 mg/kg ip) and escalating doses of morphine (10–40 mg/kg sc). On day 7, after baseline assessment, mice were given a challenge dose of morphine (10 mg/kg sc), and postinjection latencies were recorded every 30 min for 90 min. Significant tolerance developed in sham and ovx mice receiving mor and in sham and ovx mice receiving repeated MK+mor compared with mice receiving repeated saline (control). Data are presented as mean %MPE \pm SE. C and D: estimations of the AUC are presented for the tolerance experiment (A and B) as the means \pm SE. Significant tolerance developed in sham and ovx mice receiving mor and in sham and ovx mice receiving repeated MK+mor compared with mice receiving repeated saline (control). Two-way ANOVA (treatment and sex) followed by planned comparison was used for analysis. A P value of 0.05 was considered significant.

In Fig. 3, A and B, after repeated administration and a subsequent morphine challenge, there was a main effect of treatment [$F(2,79) = 17.1$; $P < 0.0001$]. In comparing mor with control mice, significant tolerance developed in sham mice [$F(2,79) = 12.5$; $P < 0.001$], and as expected, MK+mor had no effect on the development of tolerance. Significant morphine tolerance also developed in ovx mice [$F(2,79) = 13.2$; $P < 0.001$], and similar to sham, cotreatment with MK-801 had no effect on the development of tolerance (Fig. 3, A and B).

In Fig. 3, C and D, in examining AUC, there was a main effect of treatment [$F(2,79) = 18.2$; $P < 0.0001$]. In comparing mor mice with control mice, significant tolerance developed in both sham [$F(2,79) = 13.7$; $P < 0.001$] and ovx mice [$F(2,79) = 14.4$; $P < 0.001$]. In comparing mor mice with MK+mor mice, coadministration of MK-801 with morphine had no effect on the development of tolerance in either sham or ovx mice. Additionally, MK+mor mice differed significantly from control sham mice [$F(2,79) = 13.8$; $P < 0.001$] and control ovx mice [$F(2,79) = 12.8$; $P < 0.001$], indicating tolerance (Fig. 3, C and D).

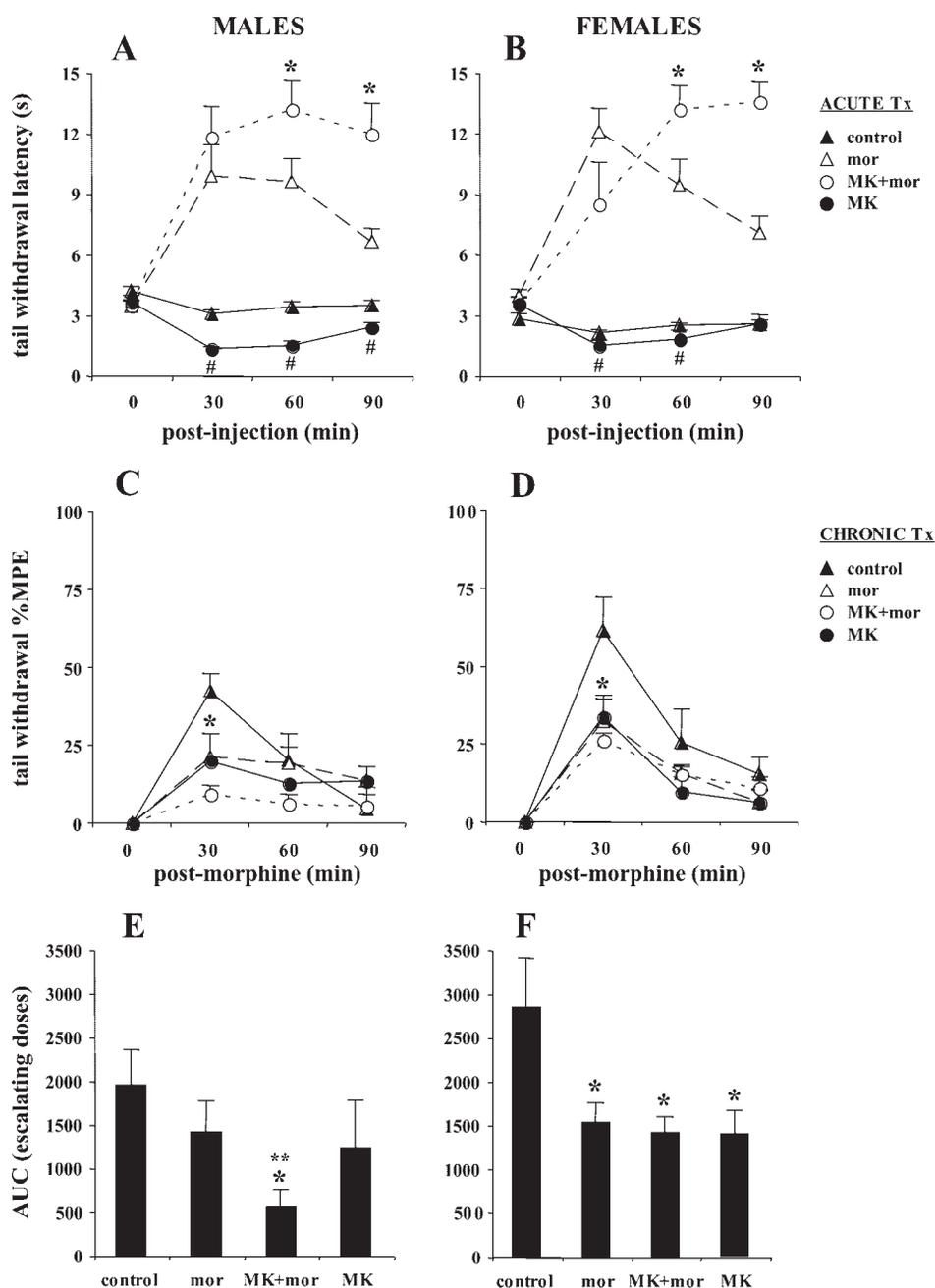
In the tail withdrawal assay, MK-801 prolongs acute morphine analgesia in male and female mice. As with the hot plate assay, in the tail withdrawal assay, MK-801 prolonged morphine analgesia in both sexes. In Fig. 4, A and B, there was a main effect of treatment [$F(3,102) = 103.1$; $P < 0.0001$] and an interaction of treatment with time [$F(6,102) = 6.6$; $P < 0.0001$]. In comparing mor with MK+mor mice, MK-801 prolonged morphine analgesia at 60 and 90 min in males [$F(6,102) = 4.6$; $P < 0.05$; $F(6,102) = 10.4$; $P < 0.01$] and females [$F(6,102) = 8.8$; $P < 0.05$; $F(6,102) = 19.2$; $P <$

0.0001]. In comparing changes in baseline latencies within subjects, both male and female mice administered MK-801 alone exhibited significant hyperalgesia at 30 and 60 min ($P < 0.05$) and in males, also at 90 min ($P < 0.05$) (Fig. 4, A and B).

In the tail withdrawal assay, after escalating doses of morphine, MK-801 facilitates the development of morphine analgesic tolerance in male, but not female, mice. In the tail withdrawal assay, MK-801 enhanced the development of morphine-induced hyperalgesia and facilitated the development of tolerance in males, but not females (Table 2, Fig. 4). In Table 2, in naïve mice, in examining changes in baseline latencies after repeated administration, there was a main effect of treatment [$F(3,80) = 12.8$; $P < 0.0001$] and sex [$F(1,80) = 16.5$; $P < 0.001$]. In comparing mor mice with control mice, morphine-induced hyperalgesia developed in males and females [$F(3,80) = 6.0$; $P < 0.05$; $F(3,80) = 9.9$; $P < 0.01$, respectively] as previously reported with this same protocol in males (11). However, in comparing mor mice with MK+mor mice, the development of morphine-induced hyperalgesia was potentiated by repeated coadministration of MK-801 in males [$F(3,80) = 4.6$; $P < 0.05$], but not females (Table 2).

In Fig. 4, C and D, after repeated administration and a subsequent morphine challenge, at 30 min, there was a main effect of treatment [$F(3,80) = 8.6$; $P < 0.0001$] and sex [$F(1,80) = 9.1$; $P < 0.01$]. In comparing mor mice with control mice, significant tolerance developed in both males [$F(3,80) = 4.8$; $P < 0.05$] and females [$F(3,80) = 8.4$; $P < 0.01$]. Furthermore, cross-tolerance developed to morphine in male and female mice receiving repeated MK-801 alone because there was a significant decrease in morphine analgesia in these mice relative to control mice (MK vs. control) [$F(3,80) = 4.9$;

Fig. 4. *A* and *B*: in the tail withdrawal assay, MK-801 prolongs acute morphine analgesia in male and female mice. After measurement of baseline hot plate latencies, male and female mice ($n = 6-8$) were administered MK-801 (1 mg/kg ip) followed immediately by morphine (7.5 mg/kg sc). Postinjection latencies were recorded every 30 min for 90 min. In comparing mice receiving acute morphine (mor) with those receiving MK+mor, there was a significant prolongation of acute morphine analgesia by MK-801 in male and female mice at 60 min and 90 min (*). Data are expressed as mean tail withdrawal latency \pm SE. #Significantly lower than baseline latency. *C* and *D*: in the tail withdrawal assay, after escalating doses of morphine, MK-801 facilitates the development of morphine tolerance in male, but not female mice. Male and female mice ($n = 10-12$) were administered MK-801 (1 mg/kg ip) and escalating doses of morphine (10–40 mg/kg sc) once daily for 6 days. On *day 7*, after baseline measurements, mice were administered a challenge dose of morphine (7.5 mg/kg sc) and tested for analgesia every 30 min for 90 min. Significant tolerance developed at 30 min postmorphine administration in males and females in comparing mice receiving mor with those receiving repeated saline (control). Furthermore, both male and female mice receiving repeated MK-801 alone (MK) exhibited cross-tolerance to morphine analgesia, as both showed a significant decrease in morphine analgesia relative to mice receiving repeated saline (control). Data are expressed as mean %MPE \pm SEM. *Significantly different from control mice. *E* and *F*: estimations of the AUC are presented for the tolerance experiment (*C* and *D*) as the mean \pm SE. In considering the entire time-effect curve, in comparing mice receiving mor with those receiving repeated saline (control), significant tolerance developed in female, but not male mice. There was a facilitation of morphine tolerance in male, but not female, mice receiving repeated MK+mor, as these mice differed significantly from mice receiving repeated saline (control) and differed marginally from mice receiving repeated mor; $**P = 0.1$. Female, but not male, mice that received repeated MK-801 alone (MK) exhibited cross-tolerance to the analgesic effect of morphine, as this group differed significantly from mice receiving repeated saline (control). *Significantly different from control mice. Two-way ANOVA (treatment and sex) followed by planned comparison was used for analysis. A P value of 0.05 was considered significant.



$P < 0.05$; $F(3,80) = 7.9$; $P < 0.01$] in the absence of any change in baseline latency (Fig. 4, *C* and *D*; Table 2).

In Fig. 4, *E* and *F*, examining AUC, there was a main effect of treatment [$F(3,80) = 5.3$; $P < 0.01$] but no effect of sex [$F(1,80) = 3.7$; $P = 0.06$]. In examining tolerance in this manner, in comparing mor mice with control mice, tolerance did not develop in males but developed in females [$F(3,80) = 6.2$; $P < 0.05$]. In male mice, the AUC of MK+mor mice was marginally less than mor mice [$F(3,80) = 2.7$; $P = 0.1$] and significantly less than control mice [$F(3,80) = 7.0$; $P < 0.01$], indicating that coadministration of MK-801 facilitated the development of morphine tolerance in males. Cross-tolerance of male mice receiving repeated MK-801 alone was not significant compared with control mice when considering the

entire time-effect curve. In females, coadministration had no effect on tolerance as MK+mor mice did not differ from mor mice, but differed from control mice [$F(3,80) = 7.0$; $P < 0.01$]. Lastly, female mice that received repeated MK-801 alone exhibited cross-tolerance to the analgesic effect of morphine, as the AUC of this group was significantly less than that of the control group receiving repeated saline [$F(3,80) = 7.9$; $P < 0.01$] (Fig. 4, *E* and *F*). This occurred in the absence of any change in baseline latency (Table 2).

DISCUSSION

In the present study, the noncompetitive NMDA receptor antagonist MK-801 prolonged acute morphine analgesia

equally in males and females in both the hot plate and tail withdrawal assays, yet at the same doses, produced a male-specific attenuation of morphine tolerance in the hot plate assay and a male-specific facilitation of tolerance in the tail withdrawal assay. Because we examined the difference in efficacy of a single challenge dose of morphine (26), it was not possible to quantify the degree of tolerance or the degree of modulation of tolerance. Thus it is possible that if tolerance were assessed in a different manner (e.g., via cumulative dose-response curves or a different morphine dose), different results might be observed.

MK-801 was previously reported to enhance acute morphine analgesia only in male mice, whereas competitive NMDA receptors enhanced morphine analgesia equally in both sexes (51). The lack of sex difference in the modulation of acute morphine analgesia in the present study is likely due to the higher dose of MK-801 that was examined (which was required to affect morphine tolerance) and/or the lower dose of morphine.

In the first report of sex differences in MK-801 modulation of morphine analgesia, there was a male-specific *attenuation* of morphine analgesia by MK-801 in deer mice (44). The discrepancy of this result with the more recent finding (51) and our study is likely due to the extremely low dose of morphine used by Lipa and Kavaliers (1 mg/kg; Ref. 44). Indeed, the noncompetitive NMDA receptor antagonist dextromethorphan produced a male-specific decrease in morphine analgesia at lower morphine doses and a male-specific enhancement at higher doses, whereas MK-801 enhanced morphine analgesia in males at all doses (51). Regardless, because our regimen produced a similar prolongation of acute morphine analgesia in both sexes and yet there was an attenuation of tolerance only in males, this suggested that the male-specific attenuation of tolerance was not simply due to a male-specific enhancement (or blockade) of acute morphine analgesia during treatment.

Robust sex differences were observed in the effect of both noncompetitive and competitive NMDA receptor antagonists on the development of morphine analgesic tolerance. In the hot plate assay, MK-801 and the competitive NMDA receptor antagonist CPP attenuated the development of morphine tolerance in male, but not female, mice (Figs. 1 and 2). Because competitive NMDA receptor antagonists were previously reported to produce equal effects on acute morphine analgesia between sexes in mice (51), this further suggested different mechanisms in NMDA receptor modulation of acute morphine analgesia and tolerance between the sexes. Additionally, in contrast to a previous report of an ovariectomy-induced reinstatement of the male-like enhancement of morphine analgesia by the noncompetitive NMDA receptor antagonist dextromethorphan in females (25), here, ovariectomy had no effect on the lack of attenuation of morphine tolerance by MK-801 (Fig. 4).

One possibility for the male-specific attenuation of morphine tolerance by MK-801 in the hot plate assay could involve motor impairment due to a number of factors, including neurobehavioral toxicity or adaptations that could oppose repeated MK-801-induced behavioral effects such as baseline hypoactivity after repeated hyperactivity (42) or changes in motor coordination after repeated ataxia (12), which could confound the hot plate response. However, in the first experiment, using lower doses of MK-801 and morphine (Fig. 1), no observable

behavioral deficits were observed during acute or chronic treatment, or at the time of testing, as previously reported (26). Additionally, after the regimen of escalating doses, on test day, we observed no visible baseline ataxia or morphine-induced ataxia in any of the groups. Lastly, the potential for male-specific impairment of motor coordination after the regimen of escalating doses seems unlikely given that, at least in rats, females are much more sensitive to the acute behavioral effects of MK-801 (30). A more plausible explanation is that females develop greater tolerance to the neurobiological effects of MK-801 on the glutamate system that are responsible for disrupting tolerance (e.g., via an upregulation of glutamate transmission or receptors).

Male and female mice (31, 37) and rats (4, 17, 35, 60) can differ in the degree of morphine tolerance. In some cases, the greater sensitivity of males to acute morphine analgesia could account for the greater tolerance observed in male rats (6) or in female rats at a particular phase of the estrous cycle (56). In the present study, the estrous cycle phase may have contributed to the variability in the magnitude of tolerance across experiments. However, the sex difference in attenuation of tolerance after NMDA receptor antagonism was not dependent on the magnitude of tolerance as represented by the AUC because females exhibited equal tolerance to males in one experiment (Fig. 1, *E* and *F*; MK-801 with constant morphine doses and the hot plate), slightly less tolerance in another experiment (Fig. 2, *C* and *D*; MK-801 with escalating morphine doses and the hot plate), and more tolerance than males in another experiment (Fig. 2, *G* and *H*; CPP with escalating doses and the hot plate), yet NMDA receptor antagonism never had an effect on the development of morphine tolerance in females. This is strong evidence that the sex difference in the effect of NMDA receptor antagonists on the development of morphine tolerance is not due to an interaction of tolerance magnitude with chronic NMDA receptor antagonism. It is still possible that either higher or lower doses of MK-801 would affect morphine tolerance in female mice. However, in male mice, a lower dose of MK-801 (0.3 mg/kg ip) had absolutely no effect on the development of morphine tolerance in the hot plate assay after escalating doses of morphine (data reported in RESULTS). We did not attempt to test higher doses in females because of the increased potential for lethality with morphine (63)

In the hot plate assay, repeated administration of MK-801 with morphine led to a reliable baseline hypoalgesia in males (Tables 1 and 2), and this was also observed in ovx females (Table 2). However, the hypothesis that baseline hypoalgesia contributes to the male-specific attenuation of morphine tolerance in the hot plate assay was not supported by the observation that neither sex demonstrated baseline hypoalgesia after repeated CPP plus morphine (Table 2), yet CPP completely blocked morphine tolerance exclusively in males. Secondly, ovariectomy reinstated the male-like baseline hypoalgesia (Table 2), yet there was no effect on morphine tolerance (Fig. 3). Thus, although males appear more susceptible to baseline hypoalgesia after repeated coadministration of MK-801 with morphine, this observation is not related to the male-specific attenuation of morphine tolerance.

In the tail withdrawal assay, MK-801 administration prolonged acute morphine analgesia similarly in both sexes (Fig. 4, *A* and *B*), yet by itself, produced hyperalgesia, as previously reported in mice using the tail pinch test (50) and in rats using

the hot plate assay (1). Furthermore, repeated MK-801 reduced the acute analgesic effect of morphine in the tail withdrawal assay but not the hot plate assay at 30 min after a morphine challenge without affecting baseline nociception at the time of testing (Fig. 4). This observation is consistent with cross-tolerance. Although we currently do not have an explanation for cross-tolerance between MK-801 and morphine, it should be noted that repeated MK-801 produces cross-sensitization to the locomotor stimulatory effect of morphine (32), highlighting at least one other opioid adaptive behavior that can be established by prior MK-801 exposure. Additionally, experimentally induced hyperalgesia can produce cross-tolerance to morphine analgesia in the absence of any change in baseline latency at the time of testing (34). Thus repeated MK-801-induced hyperalgesia could contribute to the cross-tolerance observed in both sexes in the tail withdrawal assay, perhaps via sensitization of pronociceptive processes, as observed with opioid-induced hyperalgesia (14).

In the tail withdrawal assay, there was a male-specific enhancement of the development of morphine-induced hyperalgesia by MK-801. This was associated with a male-specific facilitation of morphine tolerance (Fig. 4E). To our knowledge, there are no other studies that have examined the modulation of morphine tolerance with MK-801 using both the hot plate and tail withdrawal assays and using the same drug regimen. It is possible that the parameters could be optimized for observing attenuation of morphine tolerance using the tail withdrawal assay. Nevertheless, given the male-specific attenuation of tolerance in the hot plate assay, the male-specific facilitation in the tail withdrawal assay was unexpected and demands some consideration. First, most studies demonstrating an attenuation of morphine tolerance by MK-801 have been conducted in rats and only one report tested C57BL/6J mice. In the publication using the C57BL/6J strain, the hot plate was used to demonstrate an attenuation of morphine tolerance and not the tail flick or tail withdrawal assays (26). Here, using almost the exact same regimen, we replicated the attenuation of tolerance in the hot plate assay with males (Fig. 1). A second point is that mouse strains and rat strains differ vastly in their susceptibility to morphine tolerance (28, 36, 46), and it is likely that NMDA receptor antagonism would differentially interact with tolerance across mouse strains, which, in turn, may also depend on the pain assay. C57BL/6J mice are particularly susceptible to morphine tolerance, as evidenced by the tail withdrawal assay (36). This could explain why an attenuation of morphine tolerance by MK-801 using the tail withdrawal or tail flick assay has yet to be demonstrated in this strain. One last consideration is that a difference in stimulus intensity between the two pain assays could explain the different results. Relatedly, changing the stimulus intensity could eliminate the sex difference observed in either assay.

Chronic MK-801 administration alone can produce changes in transcription or expression of NMDA receptor subunits (41), GABA_A receptor subunits (38, 47), several genes involved in synaptic plasticity (52), striatal dopamine receptors (22, 39, 49), β -adrenergic receptors (54), and κ -receptors (10). Because of the importance of these neurotransmitter systems in baseline nociception, morphine analgesia, tolerance, and dependence, it is likely that repeated administration of MK-801 affects many pathways at several different levels of the central nervous system involved in these behaviors. Thus it is probable that

rather than chronic NMDA receptor antagonism exclusively preventing morphine-induced neuroplasticity, it induces a separate neuroplasticity that interacts with chronic morphine and presents itself differently on a behavioral level between the two pain assays (e.g., because of the difference in circuitry). Support for this hypothesis comes from the increase in baseline latency that occurs in the hot plate, but not the tail withdrawal assay after repeated administration of MK-801 alone. Second, the same treatment produces cross-tolerance in the tail withdrawal assay but not the hot plate assay (Fig. 4, E and F).

Ovariectomy in adult mice had no effect on the lack of attenuation of morphine tolerance by MK-801 in the hot plate assay (Fig. 3), which is in contrast to the modulation of acute morphine analgesia by the noncompetitive NMDA receptor antagonist dextromethorphan (25). However, because we did not monitor the estrous cycle and because chronic morphine disrupts the estrous cycle (17), it is still possible that chronic morphine decreases gonadal hormone levels in normal and sham mice, comparable to ovx mice, and this mediates the female phenotype. It is also possible that chronic MK-801 disrupts the estrous cycle in a similar manner, which could account for the lack of effect of ovariectomy. Last, male gonadectomy could result in a female phenotype, as testosterone has been shown to modulate morphine analgesia during chronic treatment (60). A complete study examining the activation and organizational effects of gonadal hormones will be necessary to determine which effects are more important in contributing to the sex difference in the modulation of morphine tolerance by NMDA receptor antagonists.

Given the lack of effect of ovariectomy on the female phenotype for morphine tolerance, it is relevant to consider other findings on sex differences related to MK-801 and morphine that similarly are not affected by ovariectomy. One report indicates that morphine causes greater activation of *c-fos*, a transcription factor associated with neuronal activation, in the intralaminar thalamic nuclei of male rats. In contrast, MK-801 causes greater activation in the nuclei of female rats, a sex difference not affected by ovariectomy (18). These nuclei are highly involved in pain processing with many spinothalamic axons terminating in this area (2). Intralaminar neurons respond to noxious stimuli (19, 55, 66) and terminate in the anterior cingulate cortex and amygdala. Both of these brain areas are involved in the motivational-affective component of pain (23, 33, 61) and contribute to the baseline hot plate response (43, 53, 65), but not the baseline tail flick response (27, 45, 53). Thus sex differences in *c-fos* activation produced by morphine and MK-801 in the intralaminar nuclei could contribute to the male-specific attenuation of morphine tolerance that was observed exclusively in the hot plate assay (e.g., via differential changes in gene expression).

In summary, NMDA receptor antagonism produces a male-specific attenuation of the development of morphine analgesic tolerance in the hot plate assay and a male-specific facilitation of tolerance in the tail withdrawal assay. This sex difference in the tolerance phenotype is most likely not related to differences in modulation of acute morphine analgesia, as both males and females showed a similar prolongation by MK-801. We conclude that male C57BL/6J mice are considerably more sensitive than female mice to perturbations in nociception and analgesia after repeated coadministration of NMDA receptor antagonists with morphine.

ACKNOWLEDGMENTS

This work was supported in part by DA05010 and AT002681. C. D. Bryant and S. Eitan were both Hatos Fellows. K. Sinchak was supported by DA13185.

REFERENCES

- Al-Amin HA, Saade NE, Khani M, Atweh S, and Jaber M. Effects of chronic dizocilpine on acute pain and on mRNA expression of neuropeptides and the dopamine and glutamate receptors. *Brain Res* 981: 99–107, 2003.
- Applebaum AE, Leonard RB, Keshalo DR Jr, Martin RF, and Willis WD. Nuclei in which functionally identified spinothalamic tract neurons terminate. *J Comp Neurol* 188: 575–585, 1979.
- Azzi L, El-Alfy M, Martel C, and Labrie F. Gender differences in mouse skin morphology and specific effects of sex steroids and dehydroepiandrosterone. *J Invest Dermatol* 124: 22–27, 2005.
- Badillo-Martinez D, Kirchgessner AL, Butler PD, and Bodnar RJ. Monosodium glutamate and analgesia induced by morphine. Test-specific effects. *Neuropharmacology* 23: 1141–1149, 1984.
- Bailey CP and Connor M. Opioids: cellular mechanisms of tolerance and physical dependence. *Curr Opin Pharmacol* 5: 60–68, 2005.
- Barrett AC, Cook CD, Turner JM, Craft RM, and Picker MJ. Importance of sex and relative efficacy at the mu opioid receptor in the development of tolerance and cross-tolerance to the antinociceptive effects of opioids. *Psychopharmacology (Berl)* 158: 154–164, 2001.
- Belozertseva IV, Dravolina OA, Neznanova ON, Danysz W, and Bespalov AY. Antinociceptive activity of combination of morphine and NMDA receptor antagonists depends on the inter-injection interval. *Eur J Pharmacol* 396: 77–83, 2000.
- Ben-Bassat J, Peretz E, and Sulman FG. Analgesimetry and ranking of analgesic drugs by the receptacle method. *Arch Int Pharmacodyn Ther* 122: 434–447, 1959.
- Bespalov A, Kudryashova M, and Zvartau E. Prolongation of morphine analgesia by competitive NMDA receptor antagonist D-CPPene (SDZ EAA 494) in rats. *Eur J Pharmacol* 351: 299–305, 1998.
- Bhargava HN, Matwyshyn GA, and Gudehithlu KP. Effects of acute and chronic administration of dizocilpine on the pharmacological responses to U-50,488H and brain and spinal cord kappa-opioid receptors in the rat. *Pharmacology* 51: 323–330, 1995.
- Bryant CD, Zaki PA, Carroll FI, and Evans CJ. Opioids and addiction: emerging pharmaceutical strategies for reducing reward and opponent processes. *Clin Neurosci Res* 5: 103–115, 2005.
- Carter AJ. Many agents that antagonize the NMDA receptor-channel complex in vivo also cause disturbances of motor coordination. *J Pharmacol Exp Ther* 269: 573–580, 1994.
- Celerier E, Laulin J, Larcher A, Le Moal M, and Simonnet G. Evidence for opiate-activated NMDA processes masking opiate analgesia in rats. *Brain Res* 847: 18–25, 1999.
- Celerier E, Laulin JP, Corcuif JB, Le Moal M, and Simonnet G. Progressive enhancement of delayed hyperalgesia induced by repeated heroin administration: a sensitization process. *J Neurosci* 21: 4074–4080, 2001.
- Chanda S, Robinette CL, Couse JF, and Smart RC. 17 β -estradiol and ICI-182780 regulate the hair follicle cycle in mice through an estrogen receptor-alpha pathway. *Am J Physiol Endocrinol Metab* 278: E202–E210, 2000.
- Craft RM and Lee DA. NMDA antagonist modulation of morphine antinociception in female vs. male rats. *Pharmacol Biochem Behav* 80: 639–649, 2005.
- Craft RM, Stratmann JA, Bartok RE, Walpole TI, and King SJ. Sex differences in development of morphine tolerance and dependence in the rat. *Psychopharmacology (Berl)* 143: 1–7, 1999.
- D'Souza DN, Harlan RE, and Garcia MM. Sexually dimorphic effects of morphine and MK-801: sex steroid-dependent and -independent mechanisms. *J Appl Physiol* 92: 493–503, 2002.
- Dostrovsky JO and Guilbaud G. Nociceptive responses in medial thalamus of the normal and arthritic rat. *Pain* 40: 93–104, 1990.
- Eddy NB and Leimbach D. Synthetic analgesics. II. Dithienylbutenyl- and dithienylbutylamines. *J Pharmacol Exp Ther* 107: 385–393, 1953.
- Eitan S, Bryant CD, Saliminejad N, Yang YC, Vojdani E, Keith D Jr, Polakiewicz R, and Evans CJ. Brain region-specific mechanisms for acute morphine-induced mitogen-activated protein kinase modulation and distinct patterns of activation during analgesic tolerance and locomotor sensitization. *J Neurosci* 23: 8360–8369, 2003.
- Gandolfi O and Dall'Olivo R. Chronic treatment with MK-801 decreases D2 dopamine receptor function in rat striatum. *Pharmacol Biochem Behav* 44: 683–687, 1993.
- Gao YJ, Ren WH, Zhang YQ, and Zhao ZQ. Contributions of the anterior cingulate cortex and amygdala to pain- and fear-conditioned place avoidance in rats. *Pain* 110: 343–353, 2004.
- Geary N, Asarian L, Korach KS, Pfaff DW, and Ogawa S. Deficits in E2-dependent control of feeding, weight gain, and cholecystokinin satiation in ER-alpha null mice. *Endocrinology* 142: 4751–4757, 2001.
- Grisel JE, Allen S, Nemmani KV, Fee JR, and Carliss R. The influence of dextromethorphan on morphine analgesia in Swiss Webster mice is sex-specific. *Pharmacol Biochem Behav* 81: 131–138, 2005.
- Hamdy MM, Noda Y, Miyazaki M, Mamiya T, Nozaki A, Nitta A, Sayed M, Assi AA, Gomaa A, and Nabeshima T. Molecular mechanisms in dizocilpine-induced attenuation of morphine dependence: an association with cortical Ca²⁺/calmodulin-dependent signal cascade. *Behav Brain Res* 152: 263–270, 2004.
- Helmstetter FJ and Bellgowan PS. Lesions of the amygdala block conditional hypoalgesia on the tail flick test. *Brain Res* 612: 253–257, 1993.
- Hoffmann O, Plesan A, and Wiesenfeld-Hallin Z. Genetic differences in morphine sensitivity, tolerance and withdrawal in rats. *Brain Res* 806: 232–237, 1998.
- Holtman JR Jr, Jing X, and Wala EP. Sex-related differences in the enhancement of morphine antinociception by NMDA receptor antagonists in rats. *Pharmacol Biochem Behav* 76: 285–293, 2003.
- Honack D and Loscher W. Sex differences in NMDA receptor-mediated responses in rats. *Brain Res* 620: 167–170, 1993.
- Hopkins E, Rossi G, and Kest B. Sex differences in systemic morphine analgesic tolerance following intrathecal morphine injections. *Brain Res* 1014: 244–246, 2004.
- Iijima Y, Asami T, and Kuribara H. Modification by MK-801 (dizocilpine), a noncompetitive NMDA receptor antagonist, of morphine sensitization: evaluation by ambulation in mice. *Nihon Shinkei Seishin Yakurigaku Zasshi* 16: 11–18, 1996.
- Johansen JP, Fields HL, and Manning BH. The affective component of pain in rodents: direct evidence for a contribution of the anterior cingulate cortex. *Proc Natl Acad Sci USA* 98: 8077–8082, 2001.
- Johnston IN and Westbrook RF. Acute and conditioned sickness reduces morphine analgesia. *Behav Brain Res* 142: 89–97, 2003.
- Kasson BG and George R. Endocrine influences on the actions of morphine: IV. Effects of sex and strain. *Life Sci* 34: 1627–1634, 1984.
- Kest B, Hopkins E, Palmese CA, Adler M, and Mogil JS. Genetic variation in morphine analgesic tolerance: a survey of 11 inbred mouse strains. *Pharmacol Biochem Behav* 73: 821–828, 2002.
- Kest B, Palmese C, and Hopkins E. A comparison of morphine analgesic tolerance in male and female mice. *Brain Res* 879: 17–22, 2000.
- Kim HS, Choi HS, Lee SY, and Oh S. Changes of GABA(A) receptor binding and subunit mRNA level in rat brain by infusion of subtoxic dose of MK-801. *Brain Res* 880: 28–37, 2000.
- Lannes B, Bernard V, Bloch B, and Micheletti G. Chronic treatment with dizocilpine maleate increases the number of striatal neurons expressing the D2 receptor gene. *Neuroscience* 65: 431–438, 1995.
- Larcher A, Laulin JP, Celerier E, Le Moal M, and Simonnet G. Acute tolerance associated with a single opiate administration: involvement of N-methyl-D-aspartate-dependent pain facilitatory systems. *Neuroscience* 84: 583–589, 1998.
- Lason W, Turchan J, Przewlocka B, Labuz D, and Przewlocki R. Effects of repeated MK-801 administration on the glutamate receptor gene expression in the rat hippocampus. *Pol J Pharmacol* 49: 249–253, 1997.
- Latsysheva NV and Rayevsky KS. Chronic neonatal N-methyl-D-aspartate receptor blockade induces learning deficits and transient hypoactivity in young rats. *Prog Neuropsychopharmacol Biol Psychiatry* 27: 787–794, 2003.
- Lee DE, Kim SJ, and Zhuo M. Comparison of behavioral responses to noxious cold and heat in mice. *Brain Res* 845: 117–121, 1999.
- Lipa SM and Kavaliers M. Sex differences in the inhibitory effects of the NMDA antagonist, MK-801, on morphine and stress-induced analgesia. *Brain Res Bull* 24: 627–630, 1990.
- Manning BH and Mayer DJ. The central nucleus of the amygdala contributes to the production of morphine antinociception in the rat tail-flick test. *J Neurosci* 15: 8199–8213, 1995.

46. **Mas M, Sabater E, Olaso MJ, Horga JF, and Faura CC.** Genetic variability in morphine sensitivity and tolerance between different strains of rats. *Brain Res* 866: 109–115, 2000.
47. **Matthews DB, Kralic JE, Devaud LL, Fritschy JM, and Marrow AL.** Chronic blockade of N-methyl-D-aspartate receptors alters gamma-aminobutyric acid type A receptor peptide expression and function in the rat. *J Neurochem* 74: 1522–1528, 2000.
48. **Micevych PE, Rissman EF, Gustafsson JA, and Sinchak K.** Estrogen receptor-alpha is required for estrogen-induced mu-opioid receptor internalization. *J Neurosci Res* 71: 802–810, 2003.
49. **Micheletti G, Lannes B, Warter JM, and Zwiller J.** Evidence for NMDA/D2 receptor-receptor interactions in the rat striatum. *Adv Neurol* 60: 128–132, 1993.
50. **Nakama-Kitamura M.** The N-Methyl-D-aspartate receptor antagonist dizocilpine inhibits associative antinociceptive tolerance to morphine in mice: relation with memory. *J Pharm Sci* 97: 75–82, 2005.
51. **Nemmani KV, Grisel JE, Stowe JR, Smith-Carliss R, and Mogil JS.** Modulation of morphine analgesia by site-specific N-methyl-D-aspartate receptor antagonists: dependence on sex, site of antagonism, morphine dose, and time. *Pain* 109: 274–283, 2004.
52. **O'Donnell J, Stemmelin J, Nitta A, Brouillette J, and Quirion R.** Gene expression profiling following chronic NMDA receptor blockade-induced learning deficits in rats. *Synapse* 50: 171–180, 2003.
53. **Pastoriza LN, Morrow TJ, and Casey KL.** Medial frontal cortex lesions selectively attenuate the hot plate response: possible nocifensive apraxia in the rat. *Pain* 64: 11–17, 1996.
54. **Paul IA, Trullas R, Skolnick P, and Nowak G.** Down-regulation of cortical beta-adrenoceptors by chronic treatment with functional NMDA antagonists. *Psychopharmacology (Berl)* 106: 285–287, 1992.
55. **Peschanski M, Guilbaud G, and Gautron M.** Posterior intralaminar region in rat: neuronal responses to noxious and nonnoxious cutaneous stimuli. *Exp Neurol* 72: 226–238, 1981.
56. **Shekunova EV and Bespalov AY.** Estrous cycle stage-dependent expression of acute tolerance to morphine analgesia in rats. *Eur J Pharmacol* 486: 259–264, 2004.
57. **Shimomura K, Shimizu H, Tsuchiya T, Abe Y, Uehara Y, and Mori M.** Is leptin a key factor which develops obesity by ovariectomy? *Endocr J* 49: 417–423, 2002.
58. **Siegel S.** Pavlovian conditioning analysis of morphine tolerance. *NIDA Res Monogr*: 27–53, 1978.
59. **Simonnet G and Rivat C.** Opioid-induced hyperalgesia: abnormal or normal pain? *Neuroreport* 14: 1–7, 2003.
60. **South SM, Wright AW, Lau M, Mather LE, and Smith MT.** Sex-related differences in antinociception and tolerance development following chronic intravenous infusion of morphine in the rat: modulatory role of testosterone via morphine clearance. *J Pharmacol Exp Ther* 297: 446–457, 2001.
61. **Tanimoto S, Nakagawa T, Yamauchi Y, Minami M, and Satoh M.** Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats. *Eur J Neurosci* 18: 2343–2350, 2003.
62. **Trujillo KA.** Are NMDA receptors involved in opiate-induced neural and behavioral plasticity? A review of preclinical studies. *Psychopharmacology (Berl)* 151: 121–141, 2000.
63. **Trujillo KA and Akil H.** The NMDA receptor antagonist MK-801 increases morphine catalepsy and lethality. *Pharmacol Biochem Behav* 38: 673–675, 1991.
64. **Vezzani A, Serafini R, Stasi MA, Caccia S, Conti I, Tridico RV, and Samanin R.** Kinetics of MK-801 and its effect on quinolinic acid-induced seizures and neurotoxicity in rats. *J Pharmacol Exp Ther* 249: 278–283, 1989.
65. **Werka T.** The effects of the medial and cortical amygdala lesions on post-stress analgesia in rats. *Behav Brain Res* 86: 59–65, 1997.
66. **Yen CT, Fu TC, and Chen RC.** Distribution of thalamic nociceptive neurons activated from the tail of the rat. *Brain Res* 498: 118–122, 1989.