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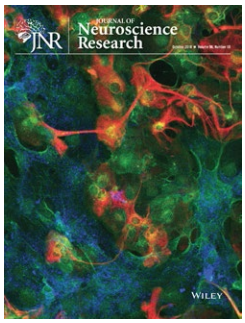

Antagonism of peripheral opioid receptors by methylnaltrexone does not prevent morphine tolerance in rats

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Abstract

Opioids are effective analgesics in the management of severe pain. However, tolerance, leading to dose escalation and adverse effects are significant limiting factors in their use. The role of peripheral opioid receptors in analgesia has been discussed especially under inflammatory conditions. The results from pharmacological and conditional knockout studies together do not provide a clear picture of the contribution of peripheral opioid receptors on antinociceptive tolerance and this needs to be evaluated. Therefore, we studied whether the peripherally restricted opioid receptor antagonist, methylnaltrexone (MNTX), could prevent morphine tolerance without attenuating the antinociceptive effect of morphine. Male Sprague-Dawley rats were treated for 7 days with increasing subcutaneous doses of morphine (5–30 mg/kg) and were coadministered saline, MNTX (0.5 or 2 mg/kg), or naltrexone (NTX; 2 mg/kg). Nociception was assessed with tail-flick, hotplate, and von Frey tests. Morphine, MNTX, and NTX concentrations in the plasma, brain, and spinal cord were measured

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by liquid chromatography-tandem mass spectrometry. In acute coadministration, NTX, but not MNTX, abolished the acute antinociceptive effects of morphine in all nociceptive tests. The antinociceptive tolerance after repeated morphine administration was also prevented by NTX but not by MNTX. MNTX penetrated to the spinal cord and the brain to some extent after repeated administration. The results do not support the use of MNTX for preventing opioid tolerance and also suggest that morphine tolerance is mediated by central rather than peripheral opioid receptors in the rat.

KEYWORDS

analgesics, drug tolerance, methylnaltrexone, morphine, naltrexone, opioid, RRID:SCR_002798

1 | INTRODUCTION

Opioids are effective analgesics in the management of acute moderate to severe pain due to trauma, surgery, or cancer (Bennett, Paice, & Wallace, 2017; Stenseth, Sellevold, & Breivik, 1985). Opioids are also essential analgesics in the management of pain at end of life. However, their use is limited by the development of tolerance leading to dose escalation and severe adverse effects such as respiratory depression, nausea, vomiting, constipation, opioid-induced hyperalgesia and dependence (Kalso, Edwards, Moore, & McQuay, 2004; Rivat & Ballantyne, 2016).

The "gold standard" opioid morphine produces antinociception mostly *via* activation of μ-opioid receptors spinally and supraspinally (Coggeshall, Zhou, & Carlton, 1997; Maldonado, Banos, & Cabanero, 2018; Mansour, Khachaturian, Lewis, Akil, & Watson, 1987; Pathan & Williams, 2012; Van Bockstaele et al., 1996). Spinal opioid receptors inhibit pain transmission directly, whereas supraspinal receptors modulate signaling via descending inhibitory pathways and engage both sensory and affective pathways, with a synergistic potentiation between these systems being reported (Ossipov et al., 2004; Sabbe & Yaksh, 1990). In addition to central opioid receptors, the significance of those located in primary afferents is discussed in opioid antinociception (Machelska & Celik, 2018; Stein, Schafer, & Machelska, 2003). Peripheral opioid receptors could provide an analgesic target with less adverse effects (Machelska & Celik, 2018). Nevertheless, the significance of peripheral opioid analgesia is still debated. In animal studies, the peripheral opioid receptors are implicated in antinociception in certain experimental pain models, especially in inflammatory and neuropathic pain (Coggeshall et al., 1997; Labuz, Mousa, Schafer, Stein, & Machelska, 2007; Mousa et al., 2017; Tiwari et al., 2016, 2018; Weibel et al., 2013). However, spinal and supraspinal opioid receptors are the main modulators of antinociception also under inflammatory conditions (Balogh et al., 2018; Corder et al., 2017; Khalefa et al., 2012).

Despite the conflicting evidence regarding the role of peripheral opioid receptors in antinociception, a recent study suggested that μ-opioid receptors in peripheral primary afferents may be important in the development of tolerance and that tolerance could be prevented by a peripherally acting opioid receptor antagonist, methylnaltrexone (MNTX), without attenuating acute antinociception (Corder et al., 2017). The possible prevention of opioid tolerance by blocking peripheral opioid receptors could significantly improve our knowledge of the opioid system and provide new possibilities in the treatment of severe pain. Studies with conditional knockout mice show that μ-opioid receptors in TRPV1- (Corder et al., 2017) or NaV1.8- (Weibel et al., 2013) positive primary afferents are not involved in antinociception in non-chronic pain models. In contrast, μ-opioid receptors in TRPV1-positive primary afferents participate in the development of opioid tolerance (Corder et al., 2017) while μ-opioid receptors in NaV1.8-positive primary afferents do not contribute to the development of tolerance (Weibel et al., 2013).

We used the peripherally restricted opioid receptor antagonist MNTX (Greenwood-Van Meerveld & Standifer, 2008) to study the role of peripheral opioid receptors in acute antinociceptive effects

Significance

Opioids are important analgesics in the management of severe pain. However, tolerance and dose escalation predispose patients to adverse effects. Recent findings have suggested that in addition to the opioid receptors located in the central nervous system, also changes in the function of peripheral opioid receptors may contribute to opioid tolerance. We show that coadministration of methylnaltrexone (MNTX), a peripherally restricted opioid antagonist, does not prevent tolerance to morphine. These results are important for the understanding of the role of peripheral opioid receptors in pain and opioid tolerance and for designing clinical pharmacodynamic-pharmacokinetic studies with MNTX.

of morphine in thermal and mechanical pain models, as well as the development of tolerance during chronic morphine administration. Because MNTX has been suggested to slowly penetrate the blood–brain barrier (Brown & Goldberg, 1985), we also measured MNTX concentrations in brain and spinal cord after repeated MNTX administration.

2 | MATERIALS AND METHODS

2.1 | Animals and ethical statement

Experiments were approved by the Southern Finland Regional State Administrative Agency (ESAVI-9697/04.10.07/2017). We followed the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and the EU2010/63 directive, adhering to the ARRIVE guidelines. Power analysis was not undertaken but group sizes were determined by the resource equation method supported by expected death of animals (Charan & Kantharia, 2013). We followed the 3R principles, minimizing the sample size, as far as reasonably achievable, for ethical reasons.

A total of 68 adult, male, pathogen-free Sprague-Dawley rats (180–250 g, Scanbur, Sollentuna, Sweden) were housed in controlled rooms (temperature 23 ± 2°C; light cycle of 12 hr). Food and water were available ad libitum. All tests were performed during the light time of the diurnal cycle. Only male rats were used to avoid hormonal influence on pain behavior. Before the experiments, animals were habituated for 5 days in the experiment room, for 2 hr daily. Animals were housed in clear plastic individually ventilated cages with two animals per cage. After the experiment, animals were anesthetized and decapitated.

2.2 | Drugs

Morphine hydrochloride powder and MNTX bromide solution (Relistor® injection, PharmaSwiss, Prague, Czech Republic) were purchased from the University Pharmacy (Helsinki, Finland). Naltrexone (NTX) hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs were dissolved in 0.9% saline and injected subcutaneously at a volume of 2 ml/kg.

2.3 | Behavioral testing

Behavioral measurements included von Frey, tail-flick, and hotplate tests. Directly after the completion of von Frey testing, rats were transferred to the tail-flick test, followed by the hot plate test. Von Frey measurements were performed using the percent response method (Deuis, Dvorakova, & Vetter, 2017). Five different filaments with ascending thickness (4, 8, 15, 26, and 60 g, North Coast Medical, Morgan Hill, CA, USA) were tested five times in ascending order and the number of responses registered. Lifting or licking the paw was registered as a response. After five responses to a single force, the rest of the filaments were registered as the maximum without further testing. In drug-naïve animals, the response rate to the thick filaments (26 and 60 g) was near maximum and decreased significantly after morphine administration. Therefore, the responses to the thick filaments were summed and the maximum effect of 10 reactions was considered as the maximum nociceptive effect. Results are presented as change from baseline (pre-post results).

Tail-flick latencies were assessed with the Ugo Basile 37360 tail-flick apparatus (Gemonio, Italy). The rat was immobilized in a plastic tube covered with a dark cloth. At each time point, latency to the flick of the tail was tested three times with 15-s intervals and the mean value calculated. The cutoff was set to 10 s to avoid tissue damage. If the cutoff value was reached, no further tests were performed for that time point.

Hotplate latencies were tested with the Ugo Basile hot/cold plate 37360 apparatus (Gemonio, Italy). The rat was placed on the hot plate (52.5°C) and the first attempt to escape or reaction to noxious heat (paw licking, paw attending, escape jumping) was registered as a response. The hotplate test was performed once at each time point and the cutoff was set to 40 s to avoid tissue damage.

2.4 | Experimental design

In the first experiment, we aimed to identify doses of MNTX that do not interfere with morphine antinociception by significant CNS penetration. The chosen MNTX doses were based on our pilot experiments (unpublished data), where MNTX doses of 0.125–2 mg/kg did not attenuate antinociception, and on an earlier publication where a 4 mg/kg intravenous bolus of MNTX produced significant brain concentrations of MNTX (Misra, Pontani, & Vadlamani, 1987). Animals were randomly allocated in two groups (n = 8 and 9) receiving morphine (4 mg/kg) with MNTX (0.5 or 2 mg/kg) subcutaneously twice a day at a 12-hr interval for 4 days and once on the morning of fifth day. At 120 min after the last drug administrations, animals were anesthetized with isoflurane (4% induction, 2.5% maintenance), a blood sample was collected in EDTA-K2 tubes on ice, and animals were transcardially perfused with phosphate-buffered saline. Plasma was separated by centrifugation (2,000 g for 10 min at +4°C). Lumbar spinal cord (L4–L6) and brain samples were collected from perfused rats. Plasma and tissue samples were snap-frozen in liquid nitrogen and stored at −80°C. Morphine, MNTX, and NTX concentrations were measured as described below.

In the following experiments, we studied the roles of central and peripheral opioid receptors in antinociception and in the development of tolerance. Animals were randomly allocated into six groups (n = 8–10). On the first day, four groups of animals received morphine (5 mg/kg) and NTX (2 mg/kg), MNTX (0.5 or 2 mg/kg), or saline. The MNTX doses were based on the first experiment.

The two remaining groups were treated with physiological saline until day 8, when one of the two received morphine. All drugs were administered subcutaneously and the treatments were given blindly according to the experimental protocol. Behavioral testing included von Frey, tail-flick, and hotplate tests before and after injections, at the time points of 30 and 90 min, on days 1, 7, and 8 of the experiment. Drug treatment was continued with another injection of morphine (5 mg/kg) or saline and the respective co-treatment after behavioral testing on the day 1 and after that every 12 hr for 7 days with increasing doses of morphine or saline as shown in Figure 1. The dosage of the antagonist (MNTX or NTX) remained unchanged during the experiment and it was administered with the same scheme as for morphine and saline. On the morning of day 7, animals received 10 mg/kg morphine or saline and the antagonist (MNTX or NTX) and the behavioral testing from the day 1 was repeated. After behavioral tests, a further dose of morphine 20 mg/kg or saline was administered with MNTX or NTX. On day 8, all groups, including one of the saline control groups, received 10 mg/kg of morphine whereas the other saline control group received saline. After the treatments, the behavioral testing was repeated and plasma was obtained for MNTX and NTX concentration analysis (14 hr after last injection of MNTX or NTX).

2.5 | Drug concentration measurements

Brain and spinal cord samples were weighed, homogenized, and dissolved in sterile water. The determinations of morphine, MNTX, and NTX concentrations were performed using a SHIMADZU UHPLC Nexera X2 (SHIMADZU USA Manufacturing inc. Canby, OR, USA) with API 3000 tandem mass spectrometry (AB Sciex, Toronto, ON, Canada) that operated in a positive turbo ion spray mode. The LC-MS/MS analyses for morphine, MNTX, and NTX were performed as previously described with minor modifications (Moreno-Vicente et al., 2015). The chromatographic separations were achieved on Atlantis HILIC Silica column (3-µm particle size, 2.1 × 100 mm I.D.; Waters, Milford, MA, USA) using a gradient elution of mobile phase consisting of acetonitrile and 20 mM ammonium acetate, pH 3.00, 90:10 (v/v). Limit of quantification (LOQ) was 0.25 ng/ml for NTX, 1 ng/ml for MNTX, and 0.5 ng/ml for morphine.

2.6 | Statistical analysis and maximum possible effect (MPE%)

Results from tail-flick and hotplate tests are presented as maximum possible effect (MPE% = [(post drug latency − baseline latency)/(cutoff − baseline latency)] × 100%). All results are presented as mean values with standard deviation. Statistical significance was analyzed by one-way ANOVA followed by Holm-Sidak's multiple comparisons test. The difference was considered significant at $p < 0.05$. Graphics and statistical analyses were made with GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA, RRID:SCR_002798).

3 | RESULTS

3.1 | Experiment 1: MNTX enters into CNS dose dependently

After the coadministration of MNTX (0.5 mg/kg or 2 mg/kg) and morphine (4 mg/kg) twice daily for 4 days and once on the day 5, we measured MNTX and morphine concentrations 120 min after the last dose. With the 0.5 mg/kg dose of MNTX, spinal cord and brain concentrations were 1.6 ng/g (4.5 pmol/g) and 0.9 ng/g (2.5 pmol/g) and, with the 2 mg/kg dose, 9.1 ng/g (25.5 pmol/g) and 5.5 ng/g (15.4 pmol/g), respectively (Figure 2a). The mean spinal cord and brain morphine concentrations were 66.5 ng/g (233 pmol/g) and 58.0 ng/g (203 pmol/g), respectively, after morphine (4 mg/kg) treatment (Figure 2b). Mean NTX concentrations were below LOQ after MNTX treatments (data not shown).

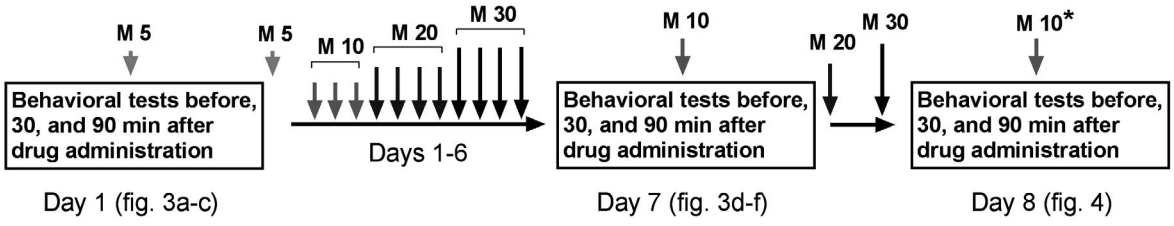

FIGURE 1 Morphine (M) dosing scheme and experimental protocol of the second experiment. Behavioral tests (von Frey, tail-flick, and hot plate; in this order) were conducted before, 30, and 90 min after drug administration on days 1, 7, and 8. On the day 1, drug-naïve animals received a test dose 5 mg/kg of morphine (M 5). After the behavioral tests, the morphine-treated animals received another dose of morphine (5 mg/kg). Thereafter, the animals received morphine with an escalating dosing schedule (10 mg/kg, M 10; 20 mg/kg, M 20; 30 mg/kg, M 30) twice a day for 6 days, twice a day. On day 7, animals received a test dose 10 mg/kg of morphine and a supplementary dose of 20 mg/kg after the behavioral tests. In the evening of day 7, animals were treated with 30 mg/kg morphine (M 30). On days 1–7, the morphine-treated animals also received saline, methylnaltrexone (0.5 or 2 mg/kg) or naltrexone (2 mg/kg) twice daily with every morphine injection. Two control groups received equivolume injections of saline. On day 8 one of the saline group received saline, while the five other groups received a 10 mg/kg test dose of morphine without any co-treatments (M 10*)

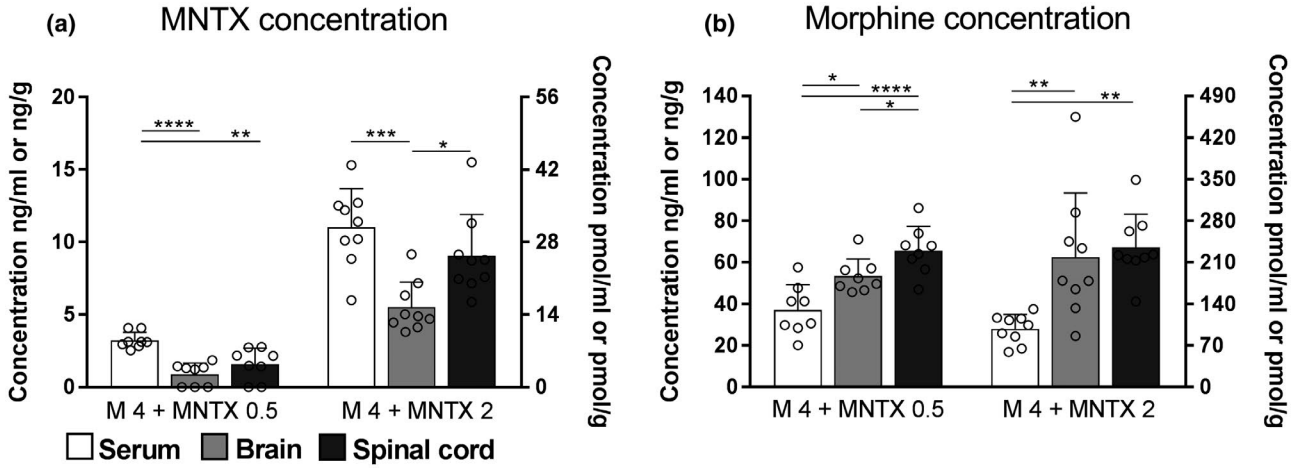

FIGURE 2 Brain, spinal cord, and plasma concentrations of methylnaltrexone (MNTX, a) and morphine (b) after repeated coadministration. Rats received MNTX (0.5 or 2 mg/kg) and morphine (4 mg/kg) twice daily for 4 days. At 120 min after the last administration, tissue samples were collected after brief transcardiac perfusion with phosphate-buffered saline. Drug concentrations measured using liquid chromatography-tandem mass spectrometry are shown as ng/ml or ng/g tissue (*Y*-axis on the left) and as pmol/ml or pmol/g tissue (*Y*-axis on the right). Statistical significance $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ (one-way ANOVA followed by Holm-Sidak's multiple comparisons test). Mean ± *SD*. $n = 8$ in the morphine + MNTX 0.5 mg/kg group and $n = 9$ in the morphine + MNTX 2 mg/kg group

3.2 | Experiment 2: Coadministration of MNTX has no effect on morphine antinociception and does not prevent morphine tolerance

Antinociceptive effects on day 1. Morphine (5 mg/kg) caused antinociception in the von Frey ($F(5, 44) = 10.59$; $n = 8$; $p = 0.0005$), tail-flick ($F(5, 44) = 60.76$; $n = 8$; $p < 0.0001$), and hotplate tests ($F(5, 44) = 6.227$; $n = 8$; $p = 0.0237$) at 30 min after administration (Figure 3a-c). Coadministration of MNTX (0.5 mg/kg or 2 mg/kg) did not attenuate the antinociceptive effect of morphine in the von Frey and tail-flick tests (Figure 3a,b). In the hotplate test, morphine produced significant antinociception despite coadministration of 2 mg/kg dose of MNTX ($p = 0.0232$), but not with the 0.5 mg/kg dose of MNTX ($p = 0.1970$) (Figure 3c). In contrast, coadministration of NTX (2 mg/kg) inhibited the effect of morphine in the von Frey ($F(5, 44) = 10.59$; $n = 8$; $p = 0.9628$), tail-flick ($F(5, 44) = 60.76$; $n = 8$; $p = 0.9927$), and hotplate ($F(5, 44) = 6.227$; $n = 8$; $p = 0.9904$) tests (Figure 3a-c). At the 90 min time-point, the results were in line with the results at 30 min, but the effect was decreasing (data not shown). The response rate for the thin filaments (4, 8, and 15 g) was already close to zero at the baseline and was not affected by acute morphine (data not shown).

3.2.1 | Antinociceptive effects on day 7

On day 7, effects of the coadministration of an opioid receptor antagonist (MNTX and NTX) with morphine were studied after chronic treatments. Morphine (10 mg/kg) had no significant antinociceptive effect in the von Frey, tail-flick, or hotplate tests (Figure 3d-f), indicating the development of tolerance. Coadministration of MNTX (0.5 or 2 mg/kg) during the morphine tolerance induction scheme failed to attenuate antinociceptive tolerance in all tests (Figure 3d-f). Also, the rats receiving NTX (2 mg/kg) along with the morphine treatment scheme did not show antinociception (Figure 3d-f). At the 90 min time-point, the results were in line with the results at 30 min, but the effect was decreasing (data not shown). The response rate for the thin von Frey filaments (4, 8 and 15 g) was already close to zero at the baseline and was not affected by either acute or chronic morphine (data not shown).

3.2.2 | Antinociceptive effects on day 8

On day 8, we examined the antinociceptive effect of morphine (10 mg/kg) without co-treatment and collected plasma for MNTX and NTX concentration analyses. Morphine (10 mg/kg) produced significant antinociception in the chronically saline-treated, drug naïve group in the von Frey ($F(5, 40) = 26.28$; $n = 8$; $p < .0001$), tail-flick ($F(5, 40) = 19.42$; $n = 8$; $p < 0.0001$), and hotplate tests ($F(5, 40) = 22.74$; $n = 8$; $p < 0.0001$) (Figure 4a-c). Morphine had a small but significant antinociceptive effect in the von Frey ($F(5, 40) = 26.28$; $n = 5$; $p = .0168$; Figure 4a), but not in the tail-flick and hotplate tests (Figure 4b,c) in chronically morphine-treated animals. Morphine (10 mg/kg) had no antinociceptive effect in the von Frey, tail-flick, and hotplate tests in either of the groups that were co-treated with morphine and MNTX (Figure 4a-c). In contrast, acute morphine (10 mg/kg) produced significant antinociception in rats that had been chronically treated with morphine and NTX (2 mg/kg) in the von Frey $F(5, 40) = 26.28$; $n = 8$; $p < 0.0001$, tail-flick $F(5, 40) = 19.42$; $n = 8$; $p < 0.0001$, and hotplate tests

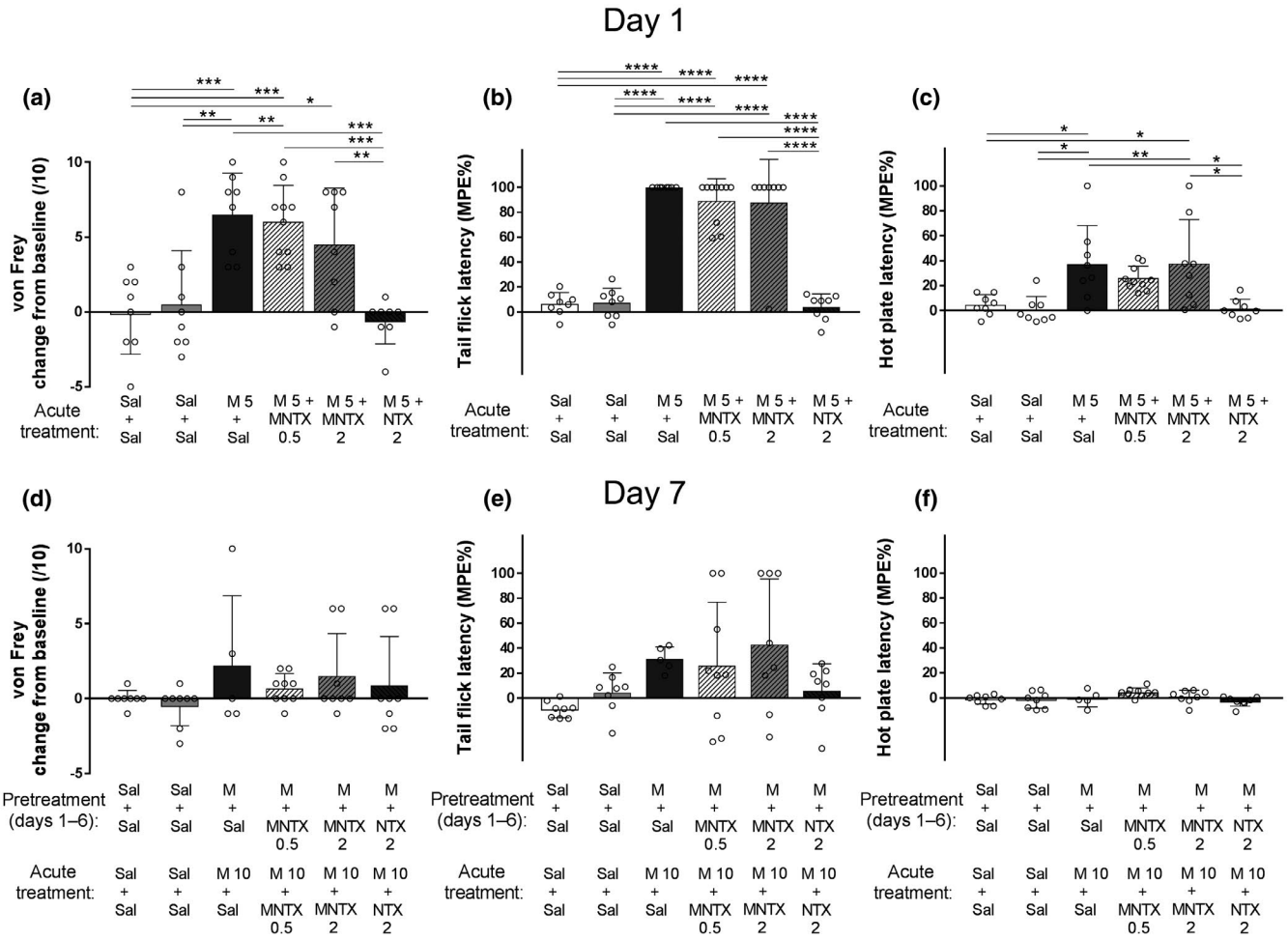

FIGURE 3 Effects of naltrexone (NTX) or methylnaltrexone (MNTX) coadministration on acute morphine (M) antinociception and the development of morphine tolerance. In the acute experiments, drug-naïve rats received subcutaneous saline (Sal) or morphine (5 mg/kg) coadministered with either saline, MNTX (0.5 or 2 mg/kg), or naltrexone (2 mg/kg). Nociceptive tests were performed 30 min after drug administrations on day 1. (a) Summed results for the 26 and 60 g von Frey filaments are presented. Maximum possible effect (MPE%) in tail-flick (b) and hotplate tests (c) are shown. After the acute administration, rats were treated with either saline (Sal) or the morphine tolerance scheme for 6 days and they also received saline, MNTX (0.5 or 2 mg/kg), or NTX (2 mg/kg). On day 7, antinociceptive effects of a morphine test dose (10 mg/kg) and the co-treatment drug (MNTX or NTX) were assessed 30 min after drug administrations. The summed results of the 26 and 60 g von Frey filaments are presented as differences from baseline (d). Maximum possible effect (MPE%) in the tail-flick (e) and hotplate (f) tests are shown. The one-way ANOVA followed by Holm-Sidak's multiple comparisons test was used. Statistical significance $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Mean ± *SD*. In panel e, two individual data points (one in the first Sal + Sal group and one in the M + MNTX 0.5 group) are below axis limits and are omitted for clarity. $n = 8$, except $n = 10$ in M + MNTX 0.5 mg/kg group (acute experiments) and $n = 8$, except $n = 9$ in the M + MNTX 0.5 mg/kg group and $n = 5$ in the M + SAL group (chronic experiments)

$F(5, 40) = 22.74$; $n = 8$; $p < 0.0001$ (Figure 4a-c). At the 90 min time-point, the results were in line with the results at 30 min, but the effect was decreasing (data not shown). The response rate for the thin filaments (4, 8, and 15 g) was already close to zero at the baseline and was not affected by either acute or chronic morphine (data not shown). MNTX and NTX concentrations in plasma were below LOQ (data not shown).

In the group that received the morphine tolerance treatment with saline, four out of nine animals died and, in morphine and MNTX (0.5 mg/kg) group, one out of 10 died during pretreatment. None of animals died in the other groups.

4 | DISCUSSION

The main findings were that the peripherally restricted opioid receptor antagonist MNTX did not affect either the acute antinociceptive effects of morphine or the development of opioid tolerance. Even moderate doses of MNTX penetrated to the CNS to some extent. In contrast, NTX inhibited the acute effects of morphine as well as the development of antinociceptive tolerance. Together, these results suggest a lack of involvement of the peripheral opioid receptors in antinociception or development of tolerance in the acute pain models studied in the rat.

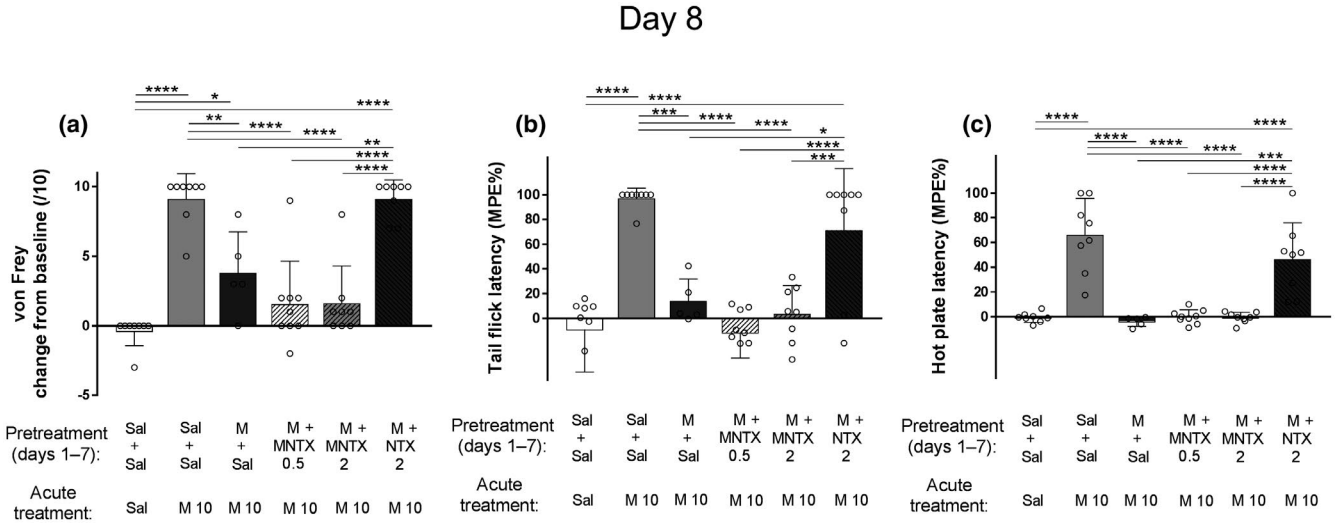

FIGURE 4 Effects of chronic coadministration of methylnaltrexone (MNTX) or naltrexone (NTX) on the development of morphine (M) tolerance as assessed by a test dose of morphine alone. Rats were pretreated with either saline (Sal) or the morphine tolerance scheme for 7 days. During this period, rats were also coadministered saline, MNTX (0.5 or 2 mg/kg) or NTX (2 mg/kg). On day 8, rats received a 10 mg/kg test dose of morphine. The summed results of the 26 and 60 g von Frey filaments are presented as a difference from baseline (a), and the maximum possible effect (MPE%) in the tail-flick (b) and hotplate (c) tests are shown. The one-way ANOVA followed by Holm-Sidak's multiple comparisons test was used. Statistical significance $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Mean ± *SD*. $n = 8$, except $n = 9$ in the M + MNTX 0.5 mg/kg group and $n = 5$ in the M + SAL group

4.1 | Brain penetration of MNTX

Although MNTX has been used as a peripherally restricted opioid receptor antagonist, earlier studies have suggested that it slowly penetrates into the brain to some extent (Brown & Goldberg, 1985; Kim, Cheng, Corrigall, & Coen, 1989). It is also demethylated to NTX in small amounts in rodents (Chandrasekaran et al., 2010; Kotake, Kuwahara, Burton, McCoy, & Goldberg, 1989; Misra et al., 1987). In the first experiment after 5 days of twice a day co-treatment with morphine, the higher dose of MNTX (2 mg/kg) produced brain and spinal cord MNTX concentrations of 15.4 nM (15.4 pmol/g) and 25.5 nM (25.5 pmol/g), respectively (Figure 2a). The MNTX concentrations were approximately sixfold lower after the 0.5 mg/kg dose, suggesting that MNTX might dose-dependently penetrate into the brain and spinal cord. Antagonism of CNS opioid receptors could also be caused by NTX, produced through demethylation of MNTX. However, we did not detect measurable NTX concentrations 120 min after MNTX administration. Morphine (4 mg/kg) concentrations were about 10-fold higher in the CNS than MNTX (2 mg/kg), suggesting that MNTX brain penetration might be about fivefold lower than for morphine in the rat after 5-day treatments. In comparison, NTX penetrates into the brain even to a greater extent than morphine (Misra, Bloch, Vardy, Mule, & Verebely, 1976). The K_i values for MNTX at the μ-opioid receptor have been reported to be 5.5 and 10 nM (Beattie et al., 2007; Kanemasa et al., 2019). After the MNTX dose of 2 mg/kg, the CNS concentrations were close to the *in vitro* K_i values for MNTX. However, in our pilot studies, MNTX did not affect the antinociceptive effects of morphine at small and moderate (0.125–2 mg/kg) doses (unpublished data). Therefore, a 2 mg/kg dose of MNTX was selected for the highest dose for the second experiment to avoid significant CNS opioid receptor antagonism. NTX was used as a positive control to produce CNS opioid receptor antagonism.

4.2 | Antagonism of peripheral opioid receptors by MNTX has no effect on acute antinociception

MNTX (0.5 and 2 mg/kg) did not affect antinociception induced by morphine in either the thermal tail-flick and hotplate tests or in the mechanical von Frey tests. Under these conditions that do not involve inflammation, CNS opioid receptors seem to mediate antinociception, as the same dose of NTX completely prevented the antinociceptive effect of morphine. These results suggest that peripheral opioid receptors do not contribute to the antinociceptive effect of morphine in these acute pain models in the rat. These results are in line with the earlier studies where small and moderate doses (below 10 mg/kg) of MNTX had no effect on antinociception (Bianchi, Fiocchi, Tavani, & Manara, 1982; Brown, Robertson, & Goldberg, 1983; Brown & Goldberg, 1985; Corder et al., 2017; Ramabadran, 1982; Russell, Bass, Goldberg, Schuster, & Merz, 1982). However, large doses (30 mg/kg) of MNTX have been reported to attenuate opioid-induced antinociception (Ramabadran, 1982; Russell et al., 1982). Also, a lower dose of 8 mg/kg of MNTX attenuated antinociception in a species- and time-dependent manner (Bianchi et al., 1982). Even though the MNTX concentrations were not measured in these studies, earlier results suggest central antagonism after large doses (Brown & Goldberg, 1985). Four out of nine animals in the morphine + saline group and one out of ten in the morphine + 0.5 mg/kg of MNTX group died during the morphine

tolerance induction protocol while no rats died in the groups receiving morphine + 2.0 mg/kg of MNTX. The most likely cause of death was respiratory depression caused by morphine, also supporting the central opioid antagonist effects of MNTX. These results may not be applicable in all pain models and conditions as peripheral opioid receptors are upregulated in inflamed tissue (Cayla et al., 2012) and described as having a significant role in local inflammatory pain (DeHaven-Hudkins et al., 1999; Labuz et al., 2007; Stein et al., 2003; Weibel et al., 2013). In conditional knockout mice, peripheral opioid receptors do not contribute to antinociception under non-inflammatory conditions but they have significance in inflammatory pain (Corder et al., 2017; Weibel et al., 2013). However, CNS receptors also seem to be the major source of antinociception also under inflammatory conditions (Khalefa et al., 2012).

4.3 | Blockade of peripheral opioid receptors by MNTX does not prevent morphine tolerance

The effect of chronic coadministration of MNTX or NTX on the development of tolerance to morphine antinociception was further studied. In line with acute studies, NTX but not MNTX cotreatment inhibited the development of tolerance, suggesting that peripheral opioid receptors do not mediate opioid tolerance in these pain models in the rat. Studies with conditional knockout mice show that μ-opioid receptors in TRPV1-positive primary afferents participate in the development of opioid tolerance but not in antinociception (Corder et al., 2017) whereas μ-opioid receptors in NaV1.8-positive neurons do not contribute to antinociception or development of tolerance (Weibel et al., 2013). With the exception of one study (Corder et al., 2017), we are not aware of earlier studies concerning MNTX and opioid tolerance. In agreement with our results, Corder et al. (2017) found that the acute antinociceptive effect of morphine was not antagonized by MNTX. However, our results do not support their findings showing attenuation of morphine tolerance by coadministration of low-dose MNTX. There were differences in several study methods such as the nociceptive tests, morphine tolerance models, and MNTX doses used. The study by Corder et al. (2017) used mice, whereas we used rats. MNTX has been reported to attenuate morphine antinociception in mice and to a lesser degree in rats, suggesting greater CNS penetration in mice (Bianchi et al., 1982; Brown & Goldberg, 1985; Russell et al., 1982). MNTX penetrates slowly into the CNS, reaching an effective concentration both time- and dose-dependently (Bianchi et al., 1982; Brown & Goldberg, 1985; Kim et al., 1989; Misra et al., 1987; Ramabadran, 1982; Russell et al., 1982), which could also explain that MNTX was efficacious only in preventing morphine tolerance. Due to slow CNS penetration of MNTX, different administration schedules and several time points for behavioral measurements should be used in future studies.

In our second study, the highest dose of MNTX used was 2 mg/kg to avoid significant CNS penetration. As we used escalating doses of morphine up to 30 mg/kg in order to induce tolerance, it is possible that the dose of 2 mg/kg MNTX may not have been high enough for complete antagonism of the possible peripheral morphine effects. However, even though the μ-opioid receptor K_i values for morphine and MNTX differ between studies, that for MNTX seems to be much lower. In previous studies, the K_i (MNTX) was seven times lower than that for morphine (Kanemasa et al., 2019; Schmidt et al., 1985), supporting our assumption that the dose we used was high enough to block peripheral effects of morphine. It is also important to note that the MNTX dose (2 mg/kg) that we used was approximately 10 times higher than that used clinically to achieve significant antagonist effects in patients treated for opioid-induced constipation (Greenwood-Van Meerveld & Standifer, 2008).

5 | CONCLUSION

In rat models of non-inflammatory pain, neither acute morphine antinociception nor development of morphine tolerance was affected by the peripherally restricted opioid antagonist MNTX, suggesting that antagonism of peripheral opioid receptors does not play a significant role in the development of opioid tolerance in the rat. Although MNTX is considered peripherally restricted, high doses lead to significant penetration into the CNS, which should be considered in future studies.

DECLARATION OF TRANSPARENCY

The authors, reviewers, and editors affirm that in accordance with the policies set by the *Journal of Neuroscience Research* this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

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CONFLICT OF INTEREST

E.K. is a member of advisory boards (Orion Pharma, Espoo, Finland, and Pierre Fabre, Toulouse, France). Other authors have no conflict of interest to report.

AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analyses. *Conceptualization:* K.B., K.D., H.V., E.K., P.R., and T.L.; *Methodology:* K.B., K.D., H.V., E.K., P.R., and T.L.; *Investigation:* K.B., K.D., J.L., P.R., and T.L.; *Formal Analysis:* K.B., K.D., P.R., and T.L.; *Resources:* E.K. and P.R.; *Writing – Original Draft:* K.B., P.R., E.K., and T.L.; *Writing – Review & Editing:* K.B., K.D., H.V., K.M., F.A., J.L., E.K.,

P.R., and T.L.; *Visualization:* K.B., E.K., P.R., and T.L.; *Supervision:* E.K., P.R., and T.L.; *Funding Acquisition:* E.K., P.R., and T.L.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Balogh, M., Zádori, Z. S., Lázár, B., Karádi, D., László, S., Mousa, S. A., … Al-Khrasani, M. (2018). The peripheral versus central antinociception of a novel opioid agonist: Acute inflammatory pain in rats. *Neurochemical Research*, *43*, 1250–1257. https://doi.org/10.1007/s11064-018-2542-7

Beattie, D. T., Cheruvu, M., Mai, N., O'Keefe, M., Johnson-Rabidoux, S., Peterson, C., … Vickery, R. (2007). The in vitro pharmacology of the peripherally restricted opioid receptor antagonists, alvimopan, ADL 08–0011 and methylnaltrexone. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *375*, 205–220. https://doi.org/10.1007/s00210-007-0146-x

Bennett, M., Paice, J. A., & Wallace, M. (2017). Pain and opioids in cancer care: Benefits, risks, and alternatives. *American Society of Clinical Oncology Educational Book*, *37*, 705–713. https://doi.org/10.14694/EDBK_180469

Bianchi, G., Fiocchi, R., Tavani, A., & Manara, L. (1982). Quaternary narcotic antagonists' relative ability to prevent antinociception and gastrointestinal transit inhibition in morphine-treated rats as an index of peripheral selectivity. *Life Sciences*, *30*, 1875–1883. https://doi.org/10.1016/0024-3205(82)90467-2

Brown, D. R., & Goldberg, L. I. (1985). The use of quaternary narcotic antagonists in opiate research. *Neuropharmacology*, *24*, 181–191. https://doi.org/10.1016/0028-3908(85)90072-3

Brown, D. R., Robertson, M. J., & Goldberg, L. I. (1983). Reversal of morphine-induced catalepsy in the rat by narcotic antagonists and their quaternary derivatives. *Neuropharmacology*, *22*, 317–321. https://doi.org/10.1016/0028-3908(83)90246-0

Cayla, C., Labuz, D., Machelska, H., Bader, M., Schafer, M., & Stein, C. (2012). Impaired nociception and peripheral opioid antinociception in mice lacking both kinin B1 and B2 receptors. *Anesthesiology*, *116*, 448–457. https://doi.org/10.1097/ALN.0b013e318242b2ea

Chandrasekaran, A., Tong, Z., Li, H., Erve, J. C., DeMaio, W., Goljer, I., … Scatina, J. (2010). Metabolism of intravenous methylnaltrexone in mice, rats, dogs, and humans. *Drug Metabolism and Disposition*, *38*, 606–616. https://doi.org/10.1124/dmd.109.031179

Charan, J., & Kantharia, N. D. (2013). How to calculate sample size in animal studies?*Journal of Pharmacology and Pharmacotherapeutics*, *4*, 303–306. https://doi.org/10.4103/0976-500X.119726

Coggeshall, R. E., Zhou, S., & Carlton, S. M. (1997). Opioid receptors on peripheral sensory axons. *Brain Research*, *764*, 126–132. https://doi.org/10.1016/S0006-8993(97)00446-0

Corder, G., Tawfik, V. L., Wang, D., Sypek, E. I., Low, S. A., Dickinson, J. R., … Scherrer, G. (2017). Loss of mu opioid receptor signaling in nociceptors, but not microglia, abrogates morphine tolerance without disrupting analgesia. *Nature Medicine*, *23*, 164–173.

DeHaven-Hudkins, D. L., Burgos, L. C., Cassel, J. A., Daubert, J. D., DeHaven, R. N., Mansson, E., … Yaksh, T. (1999). Loperamide (ADL 2–1294), an opioid antihyperalgesic agent with peripheral selectivity. *Journal of Pharmacology and Experimental Therapeutics*, *289*, 494–502.

Deuis, J. R., Dvorakova, L. S., & Vetter, I. (2017). Methods used to evaluate pain behaviors in rodents. *Frontiers in Molecular Neuroscience*, *10*, 284. https://doi.org/10.3389/fnmol.2017.00284

Greenwood-Van Meerveld, B., & Standifer, K. M. (2008). Methylnaltrexone in the treatment of opioid-induced constipation. *Clinical and Experimental Gastroenterology*, *1*, 49–58. https://doi.org/10.2147/CEG.S3889

Kalso, E., Edwards, J. E., Moore, R. A., & McQuay, H. J. (2004). Opioids in chronic non-cancer pain: Systematic review of efficacy and safety. *Pain*, *112*, 372–380. https://doi.org/10.1016/j.pain.2004.09.019

Kanemasa, T., Koike, K., Arai, T., Ono, H., Horita, N., Chiba, H., … Hasegawa, M. (2019). Pharmacologic effects of naldemedine, a peripherally acting mu-opioid receptor antagonist, in in vitro and in vivo models of opioid-induced constipation. *Neurogastroenterology and Motility*, *31*, e13563.

Khalefa, B. I., Shaqura, M., Al-Khrasani, M., Furst, S., Mousa, S. A., & Schafer, M. (2012). Relative contributions of peripheral versus supraspinal or spinal opioid receptors to the antinociception of systemic opioids. *European Journal of Pain*, *16*, 690–705. https://doi.org/10.1002/j.1532-2149.2011.00070.x

Kim, C., Cheng, R., Corrigall, W. A., & Coen, K. M. (1989). Assay for methylnalrexone in rat brain regions and serum by high-performance liquid chromatography with coulometric electrochemical detection. *Chromatographia*, *28*, 359–363.

Kotake, A. N., Kuwahara, S. K., Burton, E., McCoy, C. E., & Goldberg, L. I. (1989). Variations in demethylation of N-methylnaltrexone in mice, rats, dogs, and humans. *Xenobiotica*, *19*, 1247–1254. https://doi.org/10.3109/00498258909043176

Labuz, D., Mousa, S. A., Schafer, M., Stein, C., & Machelska, H. (2007). Relative contribution of peripheral versus central opioid receptors to antinociception. *Brain Research*, *1160*, 30–38. https://doi.org/10.1016/j.brainres.2007.05.049

Machelska, H., & Celik, M. O. (2018). Advances in achieving opioid analgesia without side effects. *Frontiers in Pharmacology*, *9*, 1388. https://doi.org/10.3389/fphar.2018.01388

Maldonado, R., Banos, J. E., & Cabanero, D. (2018). Usefulness of knockout mice to clarify the role of the opioid system in chronic pain. *British Journal of Pharmacology*, *175*, 2791–2808. https://doi.org/10.1111/bph.14088

Mansour, A., Khachaturian, H., Lewis, M. E., Akil, H., & Watson, S. J. (1987). Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain. *Journal of Neuroscience*, *7*, 2445–2464.

Misra, A. L., Bloch, R., Vardy, J., Mule, S. J., & Verebely, K. (1976). Disposition of (15,16–3H)naltrexone in the central nervous system of the rat. *Drug Metabolism and Disposition*, *4*, 276–280.

Misra, A. L., Pontani, R. B., & Vadlamani, N. L. (1987). Intravenous kinetics and metabolism of [15,16–3H]naltrexonium methiodide in the rat. *Journal of Pharmacy and Pharmacology*, *39*, 225–227. https://doi.org/10.1111/j.2042-7158.1987.tb06254.x

Moreno-Vicente, R., Fernandez-Nieva, Z., Navarro, A., Gascon-Crespi, I., Farre-Albaladejo, M., Igartua, M., … Pedraz, J. L. (2015). Development and validation of a bioanalytical method for the simultaneous determination of heroin, its main metabolites, naloxone and naltrexone by LC-MS/MS in human plasma samples: Application to a clinical trial of oral administration of a heroin/naloxone formulation. *Journal of Pharmaceutical and Biomedical Analysis*, *114*, 105–112. https://doi.org/10.1016/j.jpba.2015.04.044

Mousa, S. A., Shaqura, M., Al-Madol, M., Tafelski, S., Khalefa, B. I., Shakibaei, M., & Schafer, M. (2017). Accessibility of axonal G protein coupled mu-opioid receptors requires conceptual changes of axonal membrane targeting for pain modulation. *Journal of Controlled Release*, *268*, 352–363. https://doi.org/10.1016/j.jconrel.2017.10.016

Ossipov, M. H., Lai, J., King, T., Vanderah, T. W., Malan, T. P.Jr., Hruby, V. J., & Porreca, F. (2004). Antinociceptive and nociceptive actions of opioids. *Journal of Neurobiology*, *61*, 126–148. https://doi.org/10.1002/neu.20091

Pathan, H., & Williams, J. (2012). Basic opioid pharmacology: An update. *British Journal of Pain*, *6*, 11–16. https://doi.org/10.1177/2049463712438493

Ramabadran, K. (1982). Effects of N-methylnaloxone and N-methylnaltrexone on nociception and precipitated abstinence in mice. *Life Sciences*, *31*, 1253–1256.

Rivat, C., & Ballantyne, J. (2016). The dark side of opioids in pain management: Basic science explains clinical observation. *Pain Reports*, *1*, e570. https://doi.org/10.1097/PR9.0000000000000570

Russell, J., Bass, P., Goldberg, L. I., Schuster, C. R., & Merz, H. (1982). Antagonism of gut, but not central effects of morphine with quaternary narcotic antagonists. *European Journal of Pharmacology*, *78*, 255–261. https://doi.org/10.1016/0014-2999(82)90026-7

Sabbe, M. B., & Yaksh, T. L. (1990). Pharmacology of spinal opioids. *Journal of Pain and Symptom Management*, *5*, 191–203. https://doi.org/10.1016/0885-3924(90)90009-9

Schmidt, W. K., Tam, S. W., Shotzberger, G. S., Smith, D. H.Jr., Clark, R., & Vernier, V. G. (1985). Nalbuphine. *Drug and Alcohol Dependence*, *14*, 339–362. https://doi.org/10.1016/0376-8716(85)90066-3

Stein, C., Schafer, M., & Machelska, H. (2003). Attacking pain at its source: New perspectives on opioids. *Nature Medicine*, *9*, 1003–1008. https://doi.org/10.1038/nm908

Stenseth, R., Sellevold, O., & Breivik, H. (1985). Epidural morphine for postoperative pain: Experience with 1085 patients. *Acta Anaesthesiologica Scandinavica*, *29*, 148–156. https://doi.org/10.1111/j.1399-6576.1985.tb02176.x

Tiwari, V., Anderson, M., Yang, F., Tiwari, V., Zheng, Q., He, S. Q., … Guan, Y. (2018). Peripherally acting mu-opioid receptor agonists attenuate ongoing pain-associated behavior and spontaneous neuronal activity after nerve injury in rats. *Anesthesiology*, *128*, 1220–1236.

Tiwari, V., Yang, F., He, S. Q., Shechter, R., Zhang, C., Shu, B., … Raja, S. N. (2016). Activation of peripheral mu-opioid receptors by dermorphin [D-Arg2, Lys4] (1–4) amide leads to modality-preferred inhibition of neuropathic pain. *Anesthesiology*, *124*, 706–720.

Van Bockstaele, E. J., Colago, E. E., Cheng, P., Moriwaki, A., Uhl, G. R., & Pickel, V. M. (1996). Ultrastructural evidence for prominent distribution of the mu-opioid receptor at extrasynaptic sites on noradrenergic dendrites in the rat nucleus locus coeruleus. *Journal of Neuroscience*, *16*, 5037–5048.

Weibel, R., Reiss, D., Karchewski, L., Gardon, O., Matifas, A., Filliol, D., … Gaveriaux-Ruff, C. (2013). Mu opioid receptors on primary afferent nav1.8 neurons contribute to opiate-induced analgesia: Insight from conditional knockout mice. *PLoS ONE*, *8*, e74706. https://doi.org/10.1371/journal.pone.0074706

Zimmermann, M. (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain*, *16*, 109–110. https://doi.org/10.1016/0304-3959(83)90201-4

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