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Lack of antinociceptive cross-tolerance with co-administration of morphine and fentanyl into the periaqueductal gray of male Sprague-Dawley rats

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Abstract

Tolerance to the antinociceptive effect of mu-opioid receptor (MOPr) agonists, such as morphine and fentanyl, greatly limits their effectiveness for long-term use to treat pain. Clinical studies have shown that combination therapy and opioid rotation can be used to enhance opioid-induced antinociception once tolerance has developed. The mechanism and brain regions involved in these processes are unknown. The purpose of this study was to evaluate the contribution of the ventrolateral periaqueductal gray (vlPAG) to antinociceptive tolerance and cross-tolerance between administration and co-administration of morphine and fentanyl. Tolerance was induced by pretreating rats with morphine or fentanyl or low-dose combination of morphine and fentanyl into the vlPAG followed by assessment of cross-tolerance to the other opioid. In addition, tolerance to the combined treatment was assessed. Cross-tolerance did not develop between repeated vlPAG microinjections of morphine and fentanyl. Likewise, there was no evidence of cross-tolerance from morphine or fentanyl to co-administration of morphine and fentanyl. Co-administration did not cause cross-tolerance to fentanyl. Cross-tolerance was only evident to morphine or morphine and fentanyl combined in rats pretreated with co-administration of low-doses of morphine and fentanyl. In conclusion, cross-tolerance does not develop between morphine and fentanyl within the vlPAG. This finding is consistent with the functionally selective signaling that has been reported for antinociception and tolerance following morphine and fentanyl binding to the MOPr. This research supports the notion that combination therapy and opioid rotation may be useful clinical practices to reduce opioid tolerance and other side effects.

Perspective:

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This preclinical study shows that there is a reduction in cross tolerance between morphine and fentanyl within the periaqueductal gray which is key brain region in opioid antinociception and tolerance.

Keywords

opioid; functional selectivity; cross-tolerance; analgesia; mu-opioid receptor

Introduction

Morphine and fentanyl are two of the most commonly used drugs to treat pain. Chronic use is limited by unpleasant side effects and the development of tolerance. Opioid rotation and co-administration have been used to enhance pain relief and limit these side effects[28, 44]. Although animal studies report additive antinociceptive effects when morphine and fentanyl are co-administered[5, 39], clinical research indicates that the analgesic efficacy of co-administered morphine and fentanyl is greater than administration of either opioid alone[28, 47]. This effect appears to be the result of maintained fentanyl potency despite the development of tolerance to morphine[42].

Many preclinical studies evaluating cross-tolerance between morphine and fentanyl show enhanced antinociception and reduced tolerance when one opioid is substituted for the other[10, 35, 36, 43]. Other studies show cross-tolerance with as little as a single injection, as well as with continuous administration[26, 40]. Route and length of administration may be key factors in the analgesic efficacy of co-administered opioids.

Opioids produce antinociception by binding to mu-opioid receptors at sites throughout the nervous system. Microinjection of either morphine or fentanyl into the ventrolateral periaqueductal gray (vlPAG) produces antinociception[3] and repeated administration of either drug results in tolerance to this antinociception[1]. Despite these similarities, the intracellular signaling molecules appear to be distinct. Tolerance to repeated morphine injections into the vlPAG is mediated by C-Jun N –terminal kinase (JNK), whereas tolerance to repeated fentanyl microinjections is mediated by G protein–coupled receptor kinase (GRK)[32]. This difference suggests that within the vlPAG there should be no cross-tolerance between morphine and fentanyl microinjections. This hypothesis will be tested by microinjecting rats with morphine, fentanyl, or a combination of morphine and fentanyl directly into the vlPAG.

Methods

Subjects

Experiments were performed on male Sprague-Dawley rats (n = 93) with a mean weight of 277g (230 – 330g). Prior to surgery rats were double housed on a 12-hour light-dark cycle (lights on at 7AM). Food and water were available at all times except during testing. All procedures were approved by the Washington State University Animal Care and Use Committee and conducted in accordance with the guidelines for animal use described by the International Association for the Study of Pain.

Stereotaxic Surgery and Microinjections

Rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and implanted unilaterally with a guide cannula (23 gauge; 9 mm long) aimed at the vlPAG using stereotaxic techniques (AP: +1.7 mm, ML: ±0.6 mm, DV: −4.6 mm from lambda). Following surgery, the guide cannula was occluded with a 9 mm stylet. Rats were handled daily following surgery. Morphine sulfate (a gift from the National Institute on Drug Abuse) and fentanyl citrate (Sigma-Aldrich), were dissolved in sterile saline. Drugs were administered through a 31-gauge injection cannula inserted into and extending 2 mm beyond the guide cannula. One day prior to testing, the injector was inserted into the guide cannula without drug administration to habituate them to the procedure and prevent mechanical activation of neurons on the test day.

Behavioral testing

Nociception was assessed using the hot plate test in which the latency for the rat to lick the hind paw was measured when placed on a 52.5°C hotplate. The rat was removed if no response occurred within 50 s. Rats with a baseline hot plate latency greater than 25 s were not included in data analysis. Rats were randomly assigned and injected into the vlPAG with either 0.9% saline (0.4 μL), morphine (5 μg/0.4 μL), fentanyl (3 μg/0.4 μL), or a morphine/fentanyl combination (2.5 μg of morphine and 1.5 μg of fentanyl in 0.4 μl). These combination doses were chosen as half the ED50 dose for each opioid so as to result in an equiantinociceptive dose compared to each opioid alone. Nociception was assessed in a subset of rats at 5, 30, & 60 minutes after the first injection to determine optimal test time in tolerance experiments. Tolerance was established by repeated injections of either drug alone or the combination twice a day for two days[1]. Nociceptive testing was only conducted following the first and the last injections to prevent the development of behavioral tolerance[19]. Only male rats were used given that tolerance mediated by PAG is minimal in female rats[20].

The presence of tolerance was assessed on Day 3 using a cumulative dosing procedure[31]. Increasing third log doses of morphine (cumulative doses of 1, 2.2, 4.6, 10, 22 μg/0.4 μL), fentanyl (cumulative doses of 0.46, 1, 2.2, 4.6 & 10 μg/0.4 μL), or a combination of morphine (0.5, 1.1, 2.3, 5, & 11 μg/0.4 μL) and fentanyl (0.23, 0.5, 1.1, 2.3, & 5 μg/0.4 μL) was microinjected into the vlPAG. Half of the cumulative dose of morphine and fentanyl was used at each step when co-administered. The timing for cumulative dosing for morphine and fentanyl alone have been established previously[3, 31] as follows morphine was injected at 20 min intervals followed by hot plate testing 15 min after each injection. Fentanyl was injected at 4 min intervals with behavioral testing 2 min after each injection. Co-administered of morphine and fentanyl was injected at 7 min intervals to capture peak antinociception of the combination within the time course of both drugs (see Fig. 2). Rats were tested on the hot plate 5 min after each injection. Tolerance was defined as a significant rightward shift in the dose response curve by comparing ED50 values for rats pretreated with an opioid vs. the saline vehicle.

Histology and data analysis

Following testing, rats received a lethal dose of Halothane. Brains were removed and stored in formalin (10%). At least 2 days later the brain was sliced coronally (100 μm) to determine the location of the injection site[37]. Only those injections in or bordering the vlPAG were included in data analysis (Figure 1). Dose-response curves were plotted using GraphPad (Prism 6) and the half maximal antinociceptive effect (ED50) was calculated for each group[1]. ANOVAs were used to determine statistically significant differences between groups ($\alpha < 0.05$). Data are presented as mean ± SEM unless otherwise stated. A Bonferroni post-hoc analysis was used when necessary to compare two means.

Results

Opioid-induced antinociception in vlPAG

A subset of rats used in each of the tolerance experiments were tested before and 5, 30, and 60 minutes after opioid administration to determine the time course for antinociception to co-administration of morphine and fentanyl. There were no significant differences in baseline hot plate latencies between groups prior to drug administration ($F_{(3, 28)} = 2.24$ p = 0.11). Microinjection of morphine (5 μg/0.4 μL), fentanyl (3 μg/0.4 μL), and combined morphine/fentanyl (2.5 μg & 1.5 μg/0.4 μL) into the vlPAG caused a significant increase in hot plate latency compared to saline controls (Figure 2; $F_{(3, 143)} = 22.97$; $p < 0.05$). Administration of morphine and combined morphine/fentanyl produced antinociception at 5, 30, and 90 min post injection compared to saline controls. Microinjection of fentanyl alone had a rapid onset and offset, producing a significant increase in hot plate latency compared to saline only at the 5 min time point (Bonferroni; $p < 0.05$).

Lack of cross-tolerance between morphine and fentanyl in vlPAG

Repeated microinjections of fentanyl twice daily for 2 days did not cause a significant change in morphine potency on Day 3 compared to saline treated controls (Figure 3a; $F_{(1, 76)} = 1.66$; p = 0.20). Morphine potency was 4.2 ± 1.04 μg (N = 8) and 3.2 ± 0.96 μg (N = 8) following pretreatment with fentanyl or saline, respectively. Similarly, pretreatment with morphine did not cause a significant change in fentanyl potency (Figure 3b; $F_{(1, 71)} = 1.93$, p = 0.17). Fentanyl potency was 1.7 ± 0.67 μg (N = 7) and 2.4 ± 0.57 μg (N = 8) following pretreatment with morphine or saline, respectively. The lack of cross-tolerance between morphine and fentanyl is consistent with previous studies showing distinct intracellular mechanisms for tolerance to morphine and fentanyl antinociception[26, 32].

Co-administration of morphine and fentanyl

Co-administration of morphine and fentanyl for two days caused cross-tolerance to morphine, but not fentanyl antinociception. Pretreatment with morphine and fentanyl caused a significant rightward shift in the morphine dose-response curve compared to rats pretreated with saline (Figure 4a; $F_{(1, 66)} = 6.96$; $p < 0.05$). Morphine ED50 was 12.5 ± 3.69 μg in rats pretreated with co-administered morphine/fentanyl compared to 6.2 ± 2.35 μg in rats pretreated with saline. In contrast, co-administration of morphine and fentanyl did not alter the fentanyl dose-response curve (Figure 4b). There was no significant difference in the

antinociceptive potency of fentanyl (3.7 ± 0.52 vs. 3.9 ± 0.73) in rats pretreated with co-administered morphine/fentanyl or saline, respectively ($F_{(1, 76)} = 0.14$; p = 0.70).

Cross-tolerance was not evident when the experiment was conducted in the opposite direction. That is, pretreatment with morphine or fentanyl for two days did not cause a shift in the combined morphine/fentanyl dose-response curve (Figure 5a; $F_{(2, 114)} = 1.03$; p = 0.36). Pretreatment with morphine or fentanyl alone caused log shifts to co-administered morphine/fentanyl of only 0.07 and −1.0, respectively. However, combined pretreatment with morphine and fentanyl caused a rightward shift in the combined dose-response curve on Day 3 (Figure 5b; $F_{(1, 76)} = 9.91$; $p < 0.05$). This tolerance was evident by a full one-third log shift in the combined morphine/fentanyl ED50. This was the largest rightward shift in the dose response curve for any of the drug combinations (Table 1).

Discussion

The current study found that cross-tolerance did not develop between morphine and fentanyl when microinjected into the vlPAG using the same paradigm that produces tolerance to each drug alone[1, 31, 45]. In addition, rats treated with either opioid alone did not show tolerance to the co-administration of morphine and fentanyl. Only two conditions resulted in antinociceptive tolerance; pretreatment with low dose combination of both opioids followed by testing with the same combination or with morphine alone (Table 1).

A lack of cross-tolerance between morphine and fentanyl has also been reported following systemic administration[10, 35, 36, 43]. The clinical use of fentanyl to treat breakthrough pain in patients undergoing chronic opioid treatment also suggests a lack of cross-tolerance between fentanyl and other opioids[9, 18, 33]. Co-administration of fentanyl is frequently used to reestablish pain relief when tolerance has developed to a particular opioid[27, 47]. In addition to enhancing analgesia, co-administration of opioids has been reported to reduce side effects such as nausea, vomiting, and sedation[24, 28, 38, 41].

The lack of cross-tolerance between morphine and fentanyl suggests that these two opioids act at different sites and/or via different mechanisms. Our studies showing a lack of cross-tolerance between morphine and fentanyl when injected into the vlPAG supports the hypothesis that different mechanisms are engaged. The vlPAG plays an important role in opioid antinociception and tolerance[1, 29, 45]. In addition, the lack of cross-tolerance when rats are pretreated with a single opioid(morphine or fentanyl) and then given the co-administration also suggests distinct neural mechanisms underlie tolerance to each drug. The important implication of this experiment is that lower doses of the opioids can be used for effective antinociception after tolerance has developed to a single opioid. However, when both morphine and fentanyl are combined during pretreatment and tolerance assessment, we find tolerance does develop, likely because both morphine and fentanyl tolerance mechanisms are being activated. An interesting finding is that rats pretreated with repeated co-administration of morphine and fentanyl produces cross-tolerance to morphine alone, but not fentanyl alone. This may be attributed to the half doses that were used in the co-administration pretreatment compared to when the drugs were administered alone. It is

possible that the dose of fentanyl used in the pretreatment is inadequate to induce tolerance, whereas the dose used for morphine is sufficient to induce tolerance.

A potential contributing factor to the lack of cross-tolerance between opioids is that the affinity and efficacy at the MOPr differs between agonists. It has been shown that MOPr agonists bind and activate different splice variants of the MOPr, which may be linked to the ligand-biased effects seen in this study. Fentanyl, but not morphine antinociception is blocked following deletion of a particular exon on the MOPr, although the MOPr isoforms in the vlPAG have not been identified[34]. In addition, the formation of heterodimers (e.g., MOPr/DOPr) could contribute to downstream signaling involved in tolerance for the different opioids[8].

Morphine and fentanyl also differ in efficacy. Morphine efficacy is lower than that of fentanyl whether assessed with [^{35}S]GTPγS[23, 25, 46] or when assessing the antinociceptive effects following systemic or intrathecal administration[22, 30]. The relationship between efficacy and antinociceptive tolerance is not clear because efficacy correlates with MOPr internalization[12]. Efficacy is unlikely to have an effect on the lack of cross-tolerance reported here because we have found that morphine and fentanyl have equal antinociceptive efficacies when microinjected into the vlPAG[1].

These initial differences in receptor coupling and regulation may lead to differences in activation of signaling cascades and tolerance development. Ligand-biased signaling at the MOPr is the most likely explanation for the lack of cross-tolerance between morphine and fentanyl[25]. Morphine is typically inferior to fentanyl in inducing MOPr phosphorylation, desensitization, and internalization. Fentanyl causes phosphorylation of the MOPr via GRK, whereas morphine uses a PKC mediated mechanism[17]. In many tissue preparations morphine is very weak at inducing MOPr internalization compared to other agonists such as fentanyl[6, 7, 25, 26, 48]. This functionally selective difference in signaling has been shown to alter morphine and fentanyl antinociception. Blockade of MOPr internalization with dyn-DN had no effect on morphine antinociception, but enhanced fentanyl antinociception[2]. In contrast, inhibition of G$\alpha_{i/o}$-proteins by pertussis toxin (PTX) caused a reduction in morphine, but not fentanyl-induced antinociception[4, 14, 15].

Blockade of a component of β-arrestin signaling (i.e. G-protein receptor kinase or extracellular signal regulated kinase) has been shown to prevent tolerance to agonists, such as fentanyl, and have no effect on tolerance to morphine[2, 16, 21, 26, 32]. In contrast, inhibition of proteins downstream of G-protein signaling (i.e. protein kinase C or c-Jun n-terminal kinase) causes a reduction in morphine, but not fentanyl tolerance[16, 26, 32]. Activation of different signaling cascades would limit the development of cross-tolerance between morphine (G-protein-dependent pathway) and fentanyl (β-arrestin-dependent pathway).

The impact of differences in the duration of action between morphine and fentanyl is less clear. Fentanyl produces a rapid (3 min) and short-lived (< 30 min) antinociceptive effect compared to morphine microinjection into the vlPAG (peak effects of 15–30 min and duration of 1–2 hours)[3]. The short antinociceptive effect of fentanyl may be caused by rapid internalization, which would limit signaling through G proteins. This could explain the lack

of cross-tolerance from fentanyl to morphine, but not from morphine to fentanyl because prolonged G protein signaling by morphine should cause adaptations that affect any MOPr bound ligand.

A final difference between the two drugs is how they are metabolized. Morphine is metabolized into morphine-6-glucuronide or morphine-3-glucuronide, whereas there are no known active metabolites of fentanyl[11, 13]. The combined MOPr activation of morphine and morphine-6-glucuronide may contribute to the development of tolerance. Furthermore, morphine-3-glucuronide activation of TLR4 has been recently shown to contribute to morphine tolerance within the PAG[11]. Once again, this difference may contribute to differences in tolerance between morphine and fentanyl, but is unlikely to prevent cross-tolerance between these drugs.

In conclusion, the current study shows a clear lack of cross-tolerance between morphine and fentanyl when microinjected into the vlPAG. Although tolerance occurs with co-administration of morphine and fentanyl into the vlPAG, cross-tolerance was only evident to morphine not fentanyl. The implication of this research is that once tolerance develops to a single opioid, co-administration of lower doses of two different opioid can be co-administered to achieve antinociception. These data support clinical findings suggesting that co-administration of opioids is more effective than administration of a single opioid whether it is morphine or fentanyl. The presence of distinct tolerance mechanisms provides new targets for drug development to improve pain treatment by limiting the development of tolerance.

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Disclosures:

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HIGHLIGHTS

- The periaqueductal gray is site of action for reduced opioid cross-tolerance
- Co-administration of low-dose opioids can enhance antinociception
- Lack of cross-tolerance to opioids supports the clinical use of opioid rotation

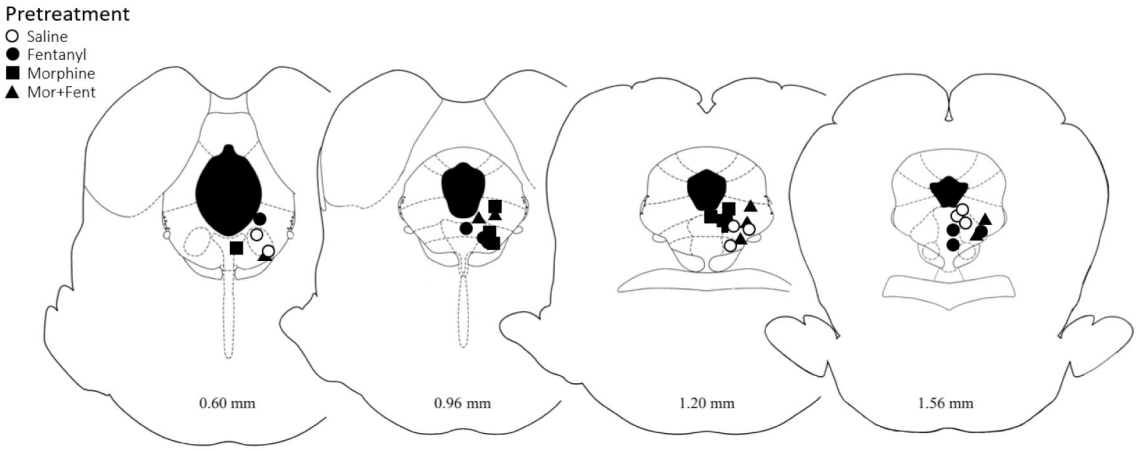

Figure 1. Location of injection sites within the vlPAG.

Cannula placements for animals pretreated with saline, morphine, fentanyl, or morphine +fentanyl. Injection sites were similar for all groups across coronal sections of the PAG. Although the image shows the location of the cannula tip, an injection volume of 0.4 μl causes the drug to diffuse into the vlPAG. Distance from Lambda are listed below each image.

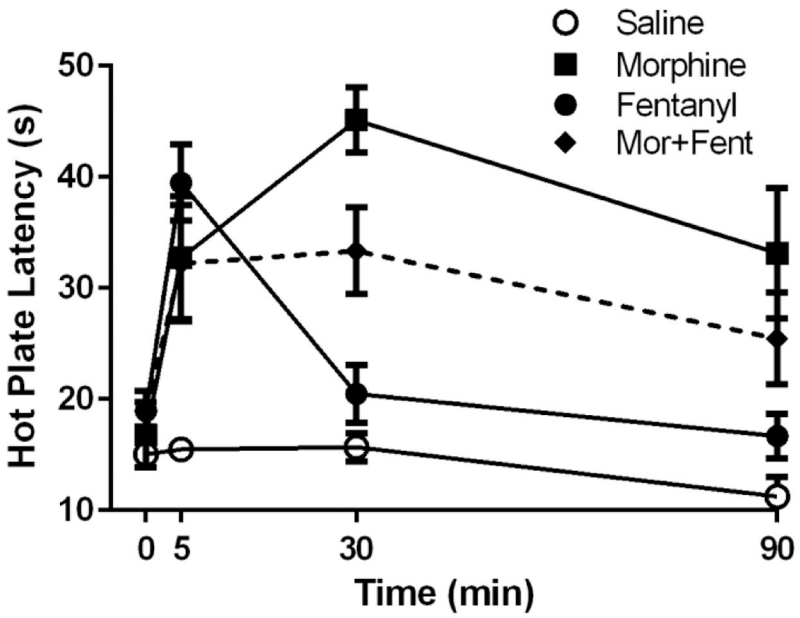

Figure 2. Time course for antinociception following vlPAG morphine, fentanyl, and co-administration of morphine and fentanyl.

Microinjection of morphine (5 μg/0.4 μL), fentanyl (3 μg/0.4 μL), and combined morphine + fentanyl (2.5 μg + 1.5 μg/0.4 μL) showed an increase in hot platency 5 min following vlPAG microinjection. Hot plate latency remained elevated for 90 min following administration of morphine (N = 8–16) or morphine and fentanyl (N = 8). In contrast, the increase in hot plate latency caused by fentanyl (N = 8–15) administration had returned to near baseline levels within 30 min. Not all rats were tested at all time points.

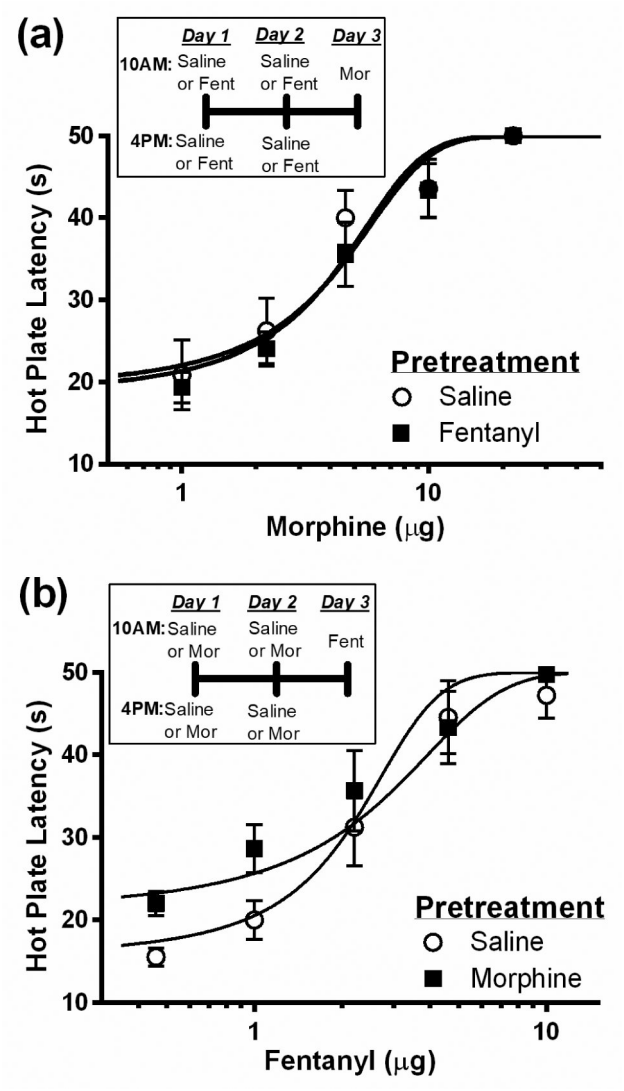

Figure 3. Lack of cross-tolerance between vlPAG morphine, and fentanyl.

Rats were injected twice daily for two days with saline (0.4 μL), morphine (5 μg/0.4 μL), or fentanyl (3 μg/0.4 μL) into the vlPAG. (a) The antinociceptive potency of morphine did not differ between rats pretreated with fentanyl (N = 8) or saline (N = 8). (b) Likewise, the antinociceptive potency of fentanyl did not differ between rats pretreated with morphine (N = 7) or saline (N = 8).

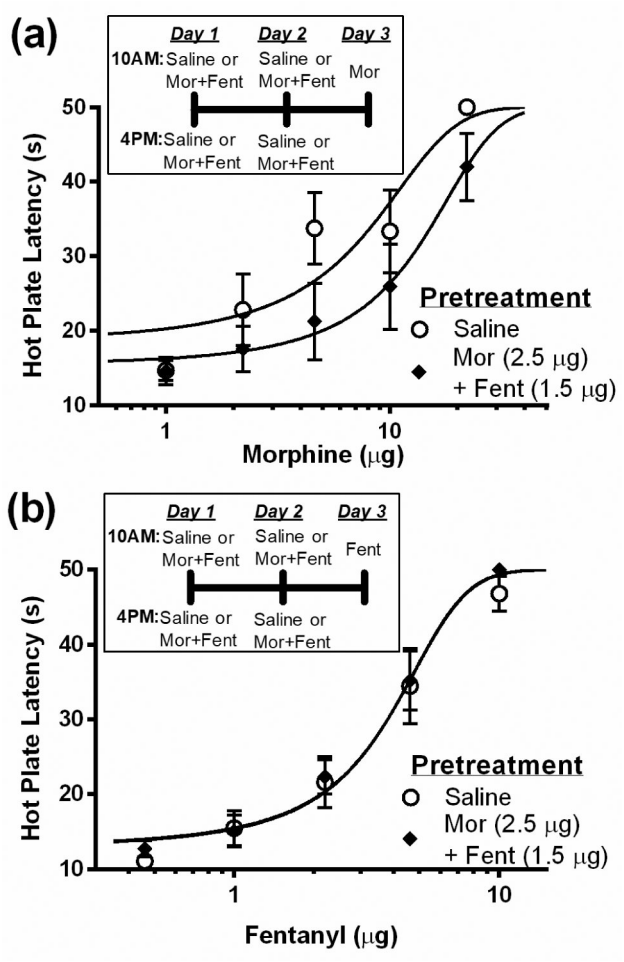

Figure 4. Co-administration of morphine and fentanyl cause cross-tolerance to morphine but not fentanyl.

(a) Repeated microinjections of morphine (2.5 μg/0.4 μL) and fentanyl (1.5 μg/0.4 μL) into the vlPAG (N = 7) for two days caused a rightward shift in the morphine dose response curve compared to saline pretreated rats (N = 7) as would be expected with the development of tolerance. (b) In contrast, co-administration of morphine and fentanyl (N = 8) had no effect on the fentanyl dose-response curve compared to rats pretreated with saline (N = 8).

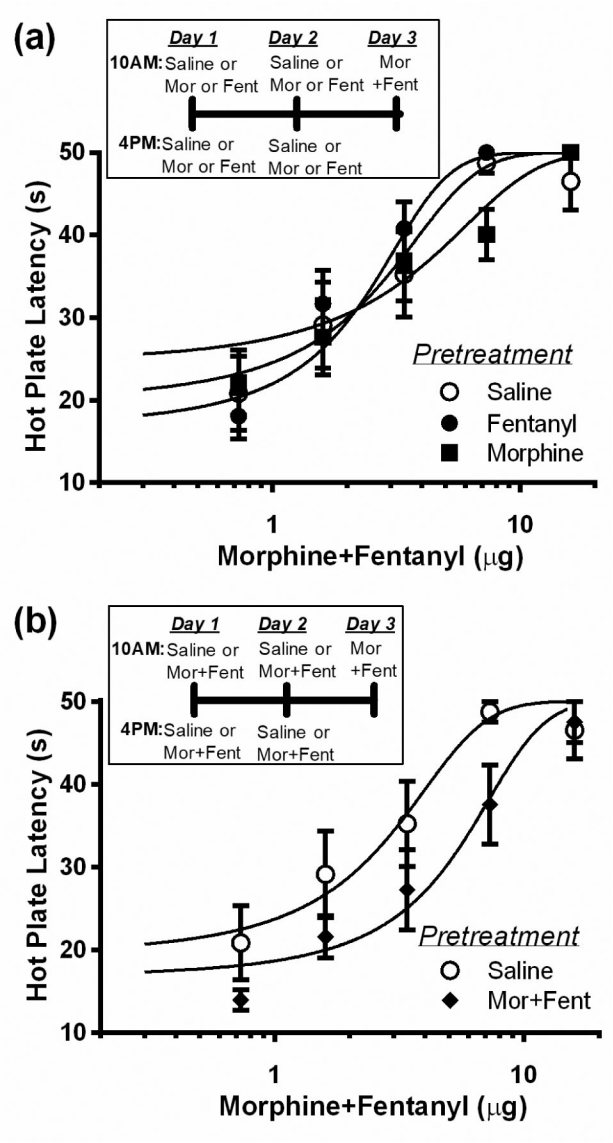

Figure 5. Lack of tolerance to morphine and fentanyl combined following pretreatment with morphine or fentanyl alone.

(a) Twice daily microinjections of morphine (5 μg/0.4 μL) or fentanyl (5 μg/0.4 μL) for two days did not cause tolerance to the combination of morphine+fentanyl. (b) Twice daily microinjections of morphine+fentanyl for two days caused a rightward shift in the combined dose-response. (N = 8/group)

Table 1:

Cross-tolerance measures as a log shift relative to saline controls

Cross-tolerance	Log shift
Fentanyl to Morphine	0.12
Morphine to Fentanyl	−0.15
Mor/Fent to Morphine	0.30 *
Mor/Fent to Fentanyl	0.02
Morphine to Mor/Fent	0.07
Fentanyl to Mor/Fent	−0.10
Mor/Fent to Mor/Fent	0.33 *

* $p < .05$