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NOP Receptor Mediates Anti-analgesia Induced by Agonist-Antagonist Opioids

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Abstract

Clinical studies have shown that agonist-antagonist opioid analgesics that produce their analgesic effect via action on the kappa-opioid receptor, produce a delayed-onset anti-analgesia in men but not women, an effect blocked by co-administration of a low dose of naloxone. We now report the same time-dependent anti-analgesia and its underlying mechanism in an animal model. Using the Randall-Selitto paw-withdrawal assay in male rats, we found that nalbuphine, pentazocine, and butorphanol each produced analgesia during the first hour followed by anti-analgesia starting at ~90 minutes after administration in males but not females, closely mimicking its clinical effects. As observed in humans, co-administration of nalbuphine with naloxone in a dose ratio of 12.5:1 blocked anti-analgesia but not analgesia. Administration of the highly selective kappa-opioid receptor agonist U69,593 produced analgesia without subsequent anti-analgesia, and confirmed by the failure of the selective kappa antagonist nor-binaltorphimine to block nalbuphine-induced anti-analgesia, indicating that anti-analgesia is not mediated by kappa-opioid receptors. We therefore tested the role of other receptors in nalbuphine anti-analgesia. Nociceptin/orphanin FQ (NOP) and sigma-1 and sigma-2 receptors were chosen on the basis of their known anti-analgesic effects and receptor binding studies. The selective NOP receptor antagonists, JTC801, and J113397, but not the sigma receptor antagonist, BD 1047, antagonized nalbuphine anti-analgesia. Furthermore, the NOP receptor agonist NNC 63-0532 produced anti-analgesia with the same delay in onset observed with the three agonist-antagonists, but without producing preceding analgesia and this anti-analgesia was also blocked by naloxone. These results strongly support the suggestion that clinically used agonist-antagonists act at the NOP receptor to produce anti-analgesia.

Keywords

κ-opioids; anti-analgesia; nalbuphine; Nociceptin/orphanin FQ receptor

Opioid analgesics, such as nalbuphine, pentazocine, and butorphanol, that have preferential action at kappa (κ)-opioid receptors with agonist-antagonist activity, are referred to as κ-

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type agonist-antagonists(Craft and McNiel, 2003; Hoskin and Hanks, 1991; Woods and Gmerek, 1985). They have been used clinically for decades, but are considered weak analgesics compared to those opioid agonists that have a much greater efficacy at the μ-opioid receptor, such as morphine or oxycodone (Levine et al., 1993; McCarthy and Lawson, 1988; McCarthy and Lawson, 1989). To better understand the variables that control the efficacy of these drugs, we conducted a series of studies in patients with post-operative pain. We found that for all three agonist-antagonists, women experience greater analgesia than men (Calderwood et al., 1988; Nobile et al., 1990; Seltzer and Shir, 1988; Wood et al., 1988). And, in a placebo controlled study, men receiving nalbuphine actually experienced worse pain than those receiving placebo (Nobile et al., 1990). Analgesia was observed in both men and women during the first hour after administration, but by ~90 minutes men reported increasing pain (i.e., anti-analgesia) (Nobile et al., 1990).

In subsequent studies we found that co-administration of nalbuphine with the non-selective opioid receptor antagonist naloxone in a narrow range of dose ratios, centered around 12.5:1 (nalbuphine : naloxone), blocks anti-analgesia and produces enhanced and prolonged analgesia in men, similar to that observed in women (Igarashi et al., 2000; Reeh et al., 1987). To explain these results, we proposed that in men nalbuphine acts at two distinct receptors, a κ-opioid “analgesia” receptor (Dodt et al., 1953) and an “anti-analgesia” receptor, the identity of which remains to be determined. Pharmacodynamic modeling recently provided support for this hypothesis (Hellon et al., 1975).

Investigating the mechanism of agonist-antagonist induced anti-analgesia using human subjects is significantly hampered by the lack of clinically available receptor selective pharmacological agents. Therefore, to take advantage of the broad range of receptor selective agents only available for animal studies, we developed an animal model of agonist-antagonist anti-analgesia in male rats. The NOP receptor was chosen because its activation at some brain sites has been associated with pain enhancement (Carpenter, 1981; Kruger et al., 1981). The sigma receptor was chosen because sigma receptors have been suggested to have anti-analgesic effects (Beitel and Dubner, 1976a; Hensel, 1981).

Experimental Procedures

Animals

All experiments were performed on 250–300 g (58 – 68 days old) adult male and female Sprague–Dawley rats (Charles River Laboratories, Hollister, CA). Animals were housed in a controlled environment at the animal care facility of the University of California, San Francisco, under a 12-h light/dark cycle. Food and water were available *ad libitum*. Experiments were approved by the Institutional Animal Care and Use Committee at UCSF and adhered to guidelines of the American Association of Laboratory Animal Care, the National Institutes of Health, and the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Effort was made to minimize the number of animals used and their suffering.

Mechanical nociceptive threshold testing

Nociceptive testing was performed using an Ugo Basile Analgesymeter (Stoelting, Chicago, IL), which applies a linearly increasing mechanical force on the dorsum of the hind paw. Nociceptive threshold was defined as the force in grams at which the rat withdrew its paw; experimenters were not blinded to the treatment. The starting pressure is 0 g and the rate of increase is 32 g/s; cut-off is 200 g, but this was not reached for any of the animals used in the current study. Each paw was treated as an independent measure and each experiment performed on a separate group of rats. Nociceptive threshold scores are obtained from a

mean of 3 measurements for each time point, taken at 5 min intervals immediately before (baseline) and after drug administration.

Prior to experiments, rats were trained in the paw-withdrawal test at 5 min intervals for 1 h/d for 3 d. On the day of the experiment, baseline paw-withdrawal threshold was measured before intravenous (i.v.) drug administration. Post-administration thresholds were recorded 30 min later and thereafter at 15 min intervals for a total of 3 h. Changes in paw-withdrawal threshold are presented as percent change from baseline.

Drugs

Nalbuphine hydrochloride, an agonist-antagonist, naloxone hydrochloride, a non-selective opioid receptor antagonist, U-69593, a selective κ-opioid receptor agonist, and nor-binaltorphimine (norBNI), a selective κ-opioid receptor antagonist were obtained from Sigma-Aldrich (St. Louis, MO). Pentazocine lactate (Talwin 30 mg/ml) was obtained from Hospira (Lake Forest, IL). Butorphanol was obtained from Bedford Laboratories (Bedford, OH). J-113397 and JTC-801, non-peptide nociceptin/orphanin FQ (NOP, ORL1) receptor selective antagonists, BD 1047, a selective sigma (σ)-receptor antagonist ($\sigma_1 > \sigma_2$), and NNC 63-0532, a selective NOP receptor agonist, were obtained from Tocris Bioscience (Ellisville, MO). SB 612111, another non-peptide selective nociceptin/orphanin FQ (NOP, ORL1) receptor antagonist, was obtained from Axon Medchem BV (Groningen, The Netherlands). Nalbuphine and naloxone were dissolved in physiological saline (0.9%); pentazocine was diluted with physiological saline to 5 mg/ml; U-69593 was dissolved in 45% aq 2-hydroxypropyl-β-cyclodextrin; J-113397, SB 612111, and JTC801 were dissolved in DMSO; and BD 1047 was dissolved in water. NNC 63-0532 was dissolved in 100% ethanol and then diluted with 0.9% saline so that the final injection was 50% ethanol; the concentration of this solution was adjusted so that the injection volume was 250 μl. Nalbuphine, pentazocine, butorphanol, naloxone, saline, U-69593, or NNC 63-0532 were administered intravenously (i.v.) into a lateral tail vein with a 25-gauge infusion catheter; animals were briefly anesthetized with 2.5% isoflurane to facilitate this procedure. To allow time for absorption J-113397, JTC801, SB 612111, and BD 1047 were administered subcutaneously (s.c.) in the nape of the neck without anesthesia 45 minutes prior to nalbuphine administration (Mizumura et al., 1991).

Receptor binding

Binding assays for nalbuphine, pentazocine, and naloxone at σ_1, σ_2, and NOP receptors were performed by MDS Pharma Services (Bothell, WA; assay services now acquired by Eurofins (https://www.eurofinspanlabs.com/Panlabs)). Details of assay binding conditions are given in Table 1.

Statistical analysis

Group data (all groups n=6) are presented as mean ± SEM; data were analyzed using one-way or two-way ANOVAs as appropriate. Significance (alpha level) was set at $p \leq 0.05$). Two-way ANOVAs demonstrating a significant interaction were further analyzed with one-way ANOVAs to determine the basis of the interaction. For within subjects effects, one way repeated measures ANOVAs were performed to determine if individual groups changed significantly over time. If so, simple contrasts were employed to determine which time points differed significantly from baseline. Because simple contrasts analysis requires multiple comparisons, a Bonferroni-type correction was applied to adjust the alpha level by dividing 0.05 by the number of comparisons. Scheffé post hoc analysis was employed to determine the basis of significance for between subjects main effects involving more than two groups.

Results

Effect of nalbuphine in males

To establish an animal model of agonist-antagonist-induced anti-analgesia, separate groups of rats received nalbuphine (0.1, 0.3, or 1.0 mg/kg) or saline (vehicle). The highest dose of nalbuphine produced analgesia early in the testing period, but, beginning at ~90 minutes, nociceptive thresholds decreased below baseline, an anti-analgesic effect that persisted to the end of the three hour experiment (Fig. 1A).

A two-way ANOVA demonstrated a significant time × group interaction ($F_{30,200}$=17.829; p<0.001), indicating that the groups responded differently over time, but not a significant main effect of group ($F_{3,20}$=2.714; p=0.072). Because the time × group interaction was significant, separate one-way repeated measures ANOVAs were performed to identify the doses of nalbuphine that produced significant change over time. There was a significant effect of time for nalbuphine 0.1 mg/kg ($F_{11,55}$=4.517; p<0.030), 0.3 mg/kg ($F_{11,55}$=23.451; p<0.001), and 1 mg/kg ($F_{11,55}$=71.399; p<0.001), but not saline ($F_{11,55}$=1.350; p=0.299). Simple contrasts examining individual time points with respect to baseline within each group, however, revealed significant differences only at the two highest nalbuphine doses.

Effect of nalbuphine in females

Earlier clinical studies showed nalbuphine-induced anti-analgesia in men but not in women (Nobile et al., 1990); therefore, we determined if this sexual dimorphism is also present in the rat. In female rats nalbuphine 1.0 mg/kg induced early analgesia but no anti-analgesia, consistent with our earlier finding in humans (Fig. 1B).

A two-way ANOVA demonstrated a significant time × group interaction ($F_{10,90}$=121.163; p<0.001), indicating that males and females responded differently over time; there was also a significant main effect of group ($F_{1,9}$=9.014; p<0.001). Because the time × group interaction was significant, a separate one-way repeated measures ANOVA for the female group showed a significant effect of time ($F_{11,55}$=4.517; p<0.030). Simple contrasts examining individual time points with respect to baseline revealed significant analgesia during the first four time points (p<0.002) but no anti-analgesia.

Effect of naloxone on nalbuphine anti-analgesia

To determine if nalbuphine anti-analgesia in the rat can be blocked by naloxone, as previously observed in humans (Reeh et al., 1987), nalbuphine (1 mg/kg, i.v.) was administered in combination with different doses of naloxone (80 μg/kg or 160 μg/kg, resulting in a nalbuphine : naloxone ratio = 12.5:1or 12.5:2, respectively). Naloxone (80 μg/kg) was administered alone as a control (Fig. 2). Both doses of naloxone blocked nalbuphine anti-analgesia, although only the lower dose of naloxone significantly prolonged its analgesia.

Two-way ANOVA showed a significant time × group interaction ($F_{20,150}$=2.125; p=0.032), and a significant main effect of group ($F_{2,15}$=14.585; p<0.001). Scheffé *post hoc* analysis showed that the analgesic effect of the 12.5:1 dose ratio was significantly greater than that of the 12.5:2 dose ratio (p=0.016). On the basis of the significant interaction term, one-way repeated measures ANOVAs were performed for each of the three groups. There was a significant main effect of time for the two groups that received both nalbuphine and naloxone ($F_{11,55}$=4.243; p=0.019 for the low dose and $F_{11,55}$=9.469; p=0.001 for the high dose naloxone group); the main effect of time for the group that received naloxone alone was not significant ($F_{11,55}$=0.682; p=0.556), indicating that naloxone itself did not have an

effect on nociceptive threshold. Simple contrasts revealed the specific time points at which the responses differed from baseline (Fig. 2).

Effect of selective κ-opioid receptor agonist and antagonist

To determine if anti-analgesia is a downstream circuit effect of κ-opioid receptor activation, we administered the selective κ-receptor agonist U69,593 (0.3 mg/kg, i.v., Fig. 3A). U69,593 at a dose that produced similar analgesia to that of nalbuphine (1 mg/kg, i.v.), did not produce anti-analgesia, indicating that anti-analgesia is unlikely to be mediated by a circuit activated by κ-receptors, either directly or through downstream activation of an anti-analgesia circuit. A one-way repeated measures ANOVA showed a significant effect of time ($F_{11,44}$=52.841; p<0.001). Simple contrasts demonstrated that the effect of U69,593 differed significantly from baseline during the first four time points, but not thereafter.

We also administered norBNI, an ultra long-lasting selective κ-opioid receptor antagonist, to test the hypothesis that nalbuphine-induced anti-analgesia was independent of an action on κ-opioid receptors. We observed that 24 h after administration of norBNI (10 mg/kg, s.c., Fig. 3B), nalbuphine induced analgesia was markedly suppressed over the first 80 min, compared to rats that received vehicle 24 h prior to nalbuphine, but that nalbuphine-induced anti-analgesia was unaffected by norBNI treatment (two-way ANOVA with Bonferroni post-hoc test showed no significant difference in the anti-analgesia phase between nalbuphine alone vs. nalbuphine + norBNI).

Role of other receptors in nalbuphine anti-analgesia

Since the anti-analgesic effect of nalbuphine is not mediated by κ-opioid receptors, we sought to generate a list of candidate anti-analgesia receptors by conducting receptor binding assays for both nalbuphine and naloxone. Samples of nalbuphine and naloxone were tested by a commercial laboratory for binding to the NOP and σ receptors (Table 1). The NOP receptor was chosen because its activation at some brain sites has been associated with pain enhancement (Carpenter, 1981; Kruger et al., 1981). Sigma receptors were chosen because they have been suggested to have anti-analgesic effects (Beitel and Dubner, 1976a; Hensel, 1981).

Role of NOP receptors

NOP receptor antagonists—To test for the involvement of the NOP receptor in nalbuphine anti-analgesia, the selective NOP receptor antagonist J-113397 (30 mg/kg, s.c.) was administered subcutaneously 45 minutes prior to nalbuphine (1 mg/kg, i.v.) and compared to the effect of the same dose of J-113397 administered 45 minutes prior to i.v. saline in a separate control group of rats. J-113397 blocked anti-analgesia prolonging nalbuphine analgesia but had no effect itself (Fig. 4A), implicating the NOP receptor as a mediator of agonist-antagonist anti-analgesia. To confirm this result, two other NOP receptor selective antagonists, SB-6112111 and JTC801, were also tested. Both similarly blocked nalbuphine anti-analgesia without affecting nociception themselves (Figs. 4B, 4C).

For J-113397 the two-way ANOVA showed a significant time × group interaction ($F_{10,100}$=9.859; p<0.001) and a significant main effect of group ($F_{1,10}$=25.471; p<0.001). Based on the significant time × group interaction, one-way repeated measures ANOVAs were performed separately for each of the groups. For the group receiving the combination of J-113397 and nalbuphine there was a significant main effect of time ($F_{11,55}$=21.125; p<0.001); simple contrasts revealed significant analgesia during the first four time points but no anti-analgesia at later time points. The main effect of time for the group receiving the combination of J-113397 and saline was not significant ($F_{11,55}$=1.286; p=0.314).

For SB-612111 the two-way ANOVA showed a significant time × group interaction ($F_{10,100}$=20.352; p<0.001) and a significant main effect of group ($F_{1,10}$=41.381; p<0.001). Based on the significant time × group interaction, one-way repeated measures ANOVAs were performed separately for each of the groups. For the group receiving the combination of SB-612111 and nalbuphine there was a significant main effect of time ($F_{11,55}$=30.995; p<0.001); simple contrasts revealed significant analgesia during the first four time points but no anti-analgesia at later time points. The main effect of time for the group receiving the combination of SB-612111 and saline was not significant ($F_{11,55}$=0.866; p=0.482).

For JTC801 the two-way ANOVA showed a significant time × group interaction ($F_{10,100}$=14.057; p<0.001) and a significant main effect of group ($F_{1,10}$=30.603; p<0.001). Based on the significant time × group interaction, one-way repeated measures ANOVAs were performed separately for each of the groups. For the group receiving the combination of JTC801 and nalbuphine there was a significant main effect of time ($F_{11,55}$=29.564; p=0.031); simple contrasts revealed significant analgesia during the first seven time points but no anti-analgesia at later time points. The main effect of time for the group receiving the combination of JTC801 and saline was also significant ($F_{11,55}$=3.377; p=0.031), but simple contrasts failed to reveal any individual time points that were significantly different from baseline.

NOP receptor agonist—To determine if selective NOP receptor activation is sufficient to produce anti-analgesia, the NOP receptor agonist NNC 63-0532 (0.3 mg/kg, i.v.) or vehicle (i.v.) as a control were administered. NNC 63-0532 produced no analgesia at any time but did produce responses significantly below baseline (i.e., demonstrating anti-analgesia) starting at ~90 minutes, similar to the anti-analgesic effects of nalbuphine (Fig. 5A).

Two-way ANOVA showed a significant group × time interaction ($F_{10,100}$= 47.058; p< 0.001) and a significant main effect of group ($F_{1,10}$= 405.590; p< 0.001). Because the time × group interaction was significant, separate one-way repeated measures ANOVAs were performed. The group receiving NNC 63-0532 showed a significant main effect of time ($F_{11,55}$=132.563; p< 0.001); the main effect of time for the group receiving vehicle was not significant ($F_{11,55}$=0.813; p= 0.520), indicating lack of change in nociceptive threshold over time. Simple contrasts for the NNC 63-0532 group showed that all time points starting with 90 minutes were significantly below baseline.

Effect of naloxone on NNC 63-0532-induced anti-analgesia—Since naloxone blocks NOP-receptor-mediated anti-analgesia induced by nalbuphine, we tested the hypothesis that naloxone would also block NNC 63-0532-induced anti-analgesia. NNC 63-0532 (0.3 mg/kg, i.v.) was administered with or without naloxone (80 μg/kg, i.v.). The group receiving NNC 63-0532 in combination with naloxone failed to show either analgesia or delayed onset anti-analgesia (Fig. 5B).

Two-way repeated measures ANOVA showed a significant group × time interaction ($F_{10,100}$= 46.830; p< 0.001) and a significant main effect of group ($F_{1,10}$= 257.473; p< 0.001). A one-way repeated measures ANOVA for the group receiving NNC 63-0532 in combination with naloxone showed that the main effect of time was not significant ($F_{11,55}$=1.952; p= 0.145), indicating lack of change in nociceptive threshold over time.

Role of σ-receptors in anti-analgesia

To test the involvement of σ-receptors in nalbuphine anti-analgesia, the σ-receptor antagonist BD 1047 was administered (10 mg/kg, s.c.) 45 minutes prior (Baccaglini and Hogan, 1983; Lee et al., 1986) to nalbuphine (1 mg/kg, i.v.). One-way repeated measures

ANOVA for this group showed a significant main effect of time ($F_{1,55}$=76.330; p<0.001). Simple contrasts revealed that both the early and late effects were significantly different from baseline, with the early time points showing analgesia and the later time points showing anti-analgesia (Fig. 6), indicating that BD 1047 did not significantly alter either the analgesic or the anti-analgesic effect of nalbuphine, thereby arguing against a role for σ-receptors in agonist-antagonist-induced anti-analgesia.

Effect of pentazocine and butorphanol in the rat

To determine if NOP-receptor-mediated anti-analgesia induced by nalbuphine is a common characteristic among κ-agonist-antagonists, the other drugs in this class, pentazocine and butorphanol, were tested in separate groups of rats. All groups received either the NOP receptor antagonist J-113397 (30 mg/kg, s.c.) or vehicle administered 45 minutes prior to testing. Both pentazocine (Fig 7A) and butorphanol (Fig. 7B) produced early analgesia followed by anti-analgesia at the later time points. As with nalbuphine and NNC 63-0532, the crossover between analgesia and anti-analgesia occurred at about 90 minutes. Also consistent with the effect of nalbuphine, J-113397 blocked the anti-analgesic effect of both pentazocine and butorphanol. These results are consistent with the receptor binding data (Table 1) showing that, like nalbuphine and naloxone, pentazocine binds to NOP receptors, and support the suggestion that κ-agonist-antagonists as a class produce anti-analgesia by acting at the NOP receptor.

Pentazocine—Two-way ANOVA showed a significant time × group interaction ($F_{10,100}$=10.206; p<0.001) and a significant main effect of group ($F_{1,10}$=68.471; p<0.001). Based on the significant time × group interaction, one-way repeated measures ANOVAs were performed separately for each group. For the group receiving pentazocine alone there was a significant main effect of time ($F_{11,55}$=68.287; p<0.001); simple contrasts revealed significant analgesia during the first 4 time points and significant anti-analgesia during the last 4 time points. The main effect of time for the group receiving the combination of J-113397 and pentazocine was also significant ($F_{11,55}$=23.325; p<0.001); simple contrasts revealed analgesia during the first 8 time points but no anti-analgesia at later time points.

Butorphanol—Two-way ANOVA showed a significant time × group interaction ($F_{10,100}$=23.841; p<0.001) but not a significant main effect of group ($F_{1,10}$=3.808; p=0.080). Based on the significant time × group interaction, one-way repeated measures ANOVAs were performed separately for each group. For the group receiving butorphanol alone there was a significant main effect of time ($F_{11,55}$=52.386; p<0.001); simple contrasts revealed significant analgesia during the first 3 time points and significant anti-analgesia during the last 3 time points. The main effect of time for the group receiving the combination of J-113397 and butorphanol was also significant ($F_{11,55}$=10.115; p=0.002); simple contrasts revealed analgesia during the first time point but no anti-analgesia at any time point.

Discussion

We found that the effects of κ-agonist-antagonists in rats closely replicate their effects in patients with postoperative pain (Nobile et al., 1990) and that the sexual dimorphism observed in humans is not species specific. Thus, as in patients, female rats demonstrated only analgesia, but agonist-antagonists produced early analgesia followed by marked anti-analgesia in males. Of note, the time at which analgesia transitioned to anti-analgesia, ~90–120 minutes after agonist-antagonist administration, was remarkably similar in both species. Furthermore, nalbuphine co-administered with naloxone at the same fixed dose ratio that was maximally effective in patients (12.5:1) blocked anti-analgesia without affecting analgesia, whereas a slightly higher dose of naloxone (dose ratio: 12.5:2) also reversed

analgesia, again closely replicating our findings in patients with postoperative pain (Reeh et al., 1987). To explain our clinical findings, we proposed that the receptor at which an agonist-antagonist acts to produce analgesia (Dodt et al., 1953) is different from the receptor at which it acts to produce anti-analgesia (Hellon et al., 1975; Igarashi et al., 2000; Reeh et al., 1987). This suggestion is supported by our current observation that the selective κ-receptor agonist U69,593 only induces analgesia, and confirmed by the failure of the selective kappa antagonist nor-BNI to block nalbuphine-induced anti-analgesia.

Based on our finding that naloxone binds to the NOP receptor, which has been implicated as pronociceptive (Hellon and Taylor, 1982), we tested for NOP receptor involvement in nalbuphine anti-analgesia. The NOP receptor antagonist J-113397 blocked nalbuphine's anti-analgesic effect, but not its analgesic effect. The same result was observed with the other two opioids used, pentazocine and butorphanol. To confirm that NOP receptor activation is sufficient to produce delayed-onset anti-analgesia, we tested two other selective NOP receptor antagonists, SB-61211 and JTC801, with nalbuphine; both blocked nalbuphine-induced anti-analgesia. Finally, we next administered the NOP receptor agonist NNC 63-0532. Although NOP receptor agonists have been shown to produce both analgesia and hyperalgesia, depending on site of injection in the central nervous system (Beitel and Dubner, 1976b; Hellon and Taylor, 1982; Iggo, 1969; Pierau and Wurster, 1981), we observed no analgesic effect of NNC 63-0532 when given systemically; rather, NNC 63-0532 mimicked nalbuphine, pentazocine, and butorphanol anti-analgesia, including a similarly delayed onset; furthermore, naloxone similarly blocked NNC 63-0532-mediated anti-analgesia. Taken together, these results strongly implicate NOP receptor activation as the basis for the anti-analgesic effects of agonist-antagonists.

The σ-receptor antagonist BD 1047 was co-administered to examine the involvement of the σ receptor, to which nalbuphine binds, in nalbuphine anti-analgesia. In a previous clinical study we investigated the involvement of the σ_1-receptor in anti-analgesia by administering the neuroleptic haloperidol, a known σ_1-receptor antagonist (Foucart et al., 1993) (BD 1047 is not approved for use in humans). Because haloperidol also binds to multiple other neurotransmitter receptors, as a control we administered chlorpromazine, which binds to many of the same receptors, but not the σ_1-receptor. Both haloperidol and chlorpromazine enhanced the analgesic effect of nalbuphine. In the current study we administered BD 1047, a σ receptor-selective ($\sigma_1 > \sigma_2$) antagonist (Szolcsanyi, 1977). BD 1047 did not significantly alter the magnitude of the analgesic or anti-analgesic effect of nalbuphine. Of note, while nalbuphine, pentazocine and naloxone had similar affinity for the NOP receptor, their binding to the σ-receptors differed markedly (see Table 1), further ruling out a role for σ-receptors in the naloxone sensitive anti-analgesia induced by nalbuphine and pentazocine. The anti-analgesia effect of nalbuphine, pentazocine and butorphanol is a NOP receptor-dependent phenomenon, since the NOP receptor antagonists, J-112297, JTC-801 and SB-612111, completely block anti-analgesia. That the binding data for nalbuphine and pentazocine shows only weak binding for the NOP receptor, raises the question as to whether this level of binding would be sufficient to produce the observed anti-analgesia. An alternative mechanism of action is that the kappa opioid agonist-antagonists act indirectly to release an endogenous nociceptin/orphanin FQ ligand. However, such an effect of the opioid agonist-antagonists could not be via action at the kappa opioid receptor, since the selective kappa agonists U69,593 and U50,488 do not produce anti-analgesia; we are not aware of a non-kappa, non-nociceptin/orphanin FQ receptor through which these three opioid agonist-antagonists could act to release an endogenous ligand. Of note, while another agonist-antagonist opioid, buprenorphine, also has weak *in vitro* binding and activity at the NOP receptor (Khroyan et al., 2009), it does have significant *in vivo* activity, which has been suggested to be due to its lipophilicity and slow receptor dissociation rate (Lewis, 1985).

Thus, it remains to be determined whether the NOP-mediated anti-analgesic effect produced by the agonist-antagonist opioids is a direct or indirect effect on the NOP receptor.

A number of previous studies in humans from other laboratories (Calderwood and Hahn, 1983; Gazda et al., 2001), in primates (Mizumura et al., 1994), and in rodents (Amann, 1990; Greenlund et al., 1995; Homma et al., 2002; Pierau et al., 1975) have examined the analgesic effects of those drugs that act on the κ-opioid receptor, including agonist-antagonists, but none were able to detect the anti-analgesic effect of κ-opioid-preferring agonist-antagonists or other κ-opioid-preferring agonists. Importantly, the current study sheds light on these apparent discrepancies. First, highly selective κ-opioid receptor agonists such as U69,593, or U50,488, which were tested in some of these studies, would not be expected to induce NOP-receptor mediated anti-analgesia. Second, agonist-antagonist anti-analgesia is a delayed effect. Our clinical studies (Calderwood et al., 1988; Nobile et al., 1990), our pharmacodynamic modeling (Hellon et al., 1975), and the current study in the rat show that the onset of anti-analgesia is more than an hour after administration of the agonist-antagonist; with the exception of Khasar et al., none of the previous studies tested the effects of agonist-antagonists at time point that would reveal the late onset anti-analgesia. And while Khasar et al. evaluated nociceptive thresholds at different time points, up to 120 min post-nalbuphine administration the data were collapsed over time, i.e. an area under the curve analysis was performed to provide a single value for each dose of nalbuphine. Thus, given the initial antinociception following nalbuphine administration, integrating nociceptive threshold over 120 min evaluation period would obscure any anti-analgesia occurring in the latter portion of this period.

While the present study reveals NOP as the receptor at which the agonist-antagonists act to produce anti-analgesia, the basis of the long delay (>1 hour) in onset of the anti-analgesia (by either nalbuphine, pentazocine, butorphanol, or the selective NOP-receptor agonist NNC 63-0532) remains to be determined. It is, however, unlikely to be a pharmacokinetic effect of κ-opioid preferring agonist-antagonists, since nalbuphine and pentazocine analgesia have a short latency to onset, and NNC 63-0532 shows the same latency to onset of anti-analgesia as the agonist-antagonists, without producing prior analgesia. It is possible that the delayed effect of onset of the anti-analgesic action is a result of it being mediated by metabolites of the administered drugs. However, this is unlikely as while drug metabolites of nalbuphine, pentazocine and butorphanol may have pharmacological activity that differs from that of their parent compound, if the anti-analgesia was being produced by their metabolites, this would mean that the metabolites of two different classes of opioids, phenanthrene and benzomophan, both activate NOP receptors to produce anti-analgesia. In addition, the lack of nalbuphine anti-analgesia in female rats would mean a different metabolic pathway for nalbuphine in females. While there is no evidence for such a sex dimorphism in drug metabolism, there is a sexual dimorphism of NOP receptor-mediated effects. For example, it has been shown that orphanin FQ produces gender-specific modulation of nociception (Flores et al., 2001) an effect that is sex hormone dependent (Claiborne et al., 2006; Kopec and Sayre, 2004). In addition, there are sex differences in the distribution of the NOP receptor in the trigeminal nucleus caudalis, and it appears to be both co-localized with the estrogen receptor and estrogen levels modulate ORL1 receptor mRNA (Flores et al., 2003).

In conclusion we have shown that the κ-opioid receptor preferring agonist-antagonist class of opioids produces analgesic and anti-analgesic effects in rats similar to those observed in humans, supporting the rat as a model for the study of this class of opioids. We also found that anti-analgesia can be demonstrated in the rat and that this effect does not result from action at the κ-opioid receptor, but rather is NOP receptor-mediated. These findings help to explain previous conflicting results in which studies in animal and human experimental pain

models were unable to detect these differences and should facilitate novel strategies for development of more effective members of this class of analgesic drugs.

Acknowledgments

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Highlights

- In male rats, κ-opioids produce analgesia, followed by anti-analgesia.
- Low-dose naloxone blocked anti-analgesia but not analgesia.
- Nociceptin/orphanin FQ (NOP) antagonists also blocked anti-analgesia.
- Selective kappa antagonist nor-BNI did not block nalbuphine-induced anti-analgesia.
- Clinically used agonist-antagonists act at NOP receptors to produce anti-analgesia.

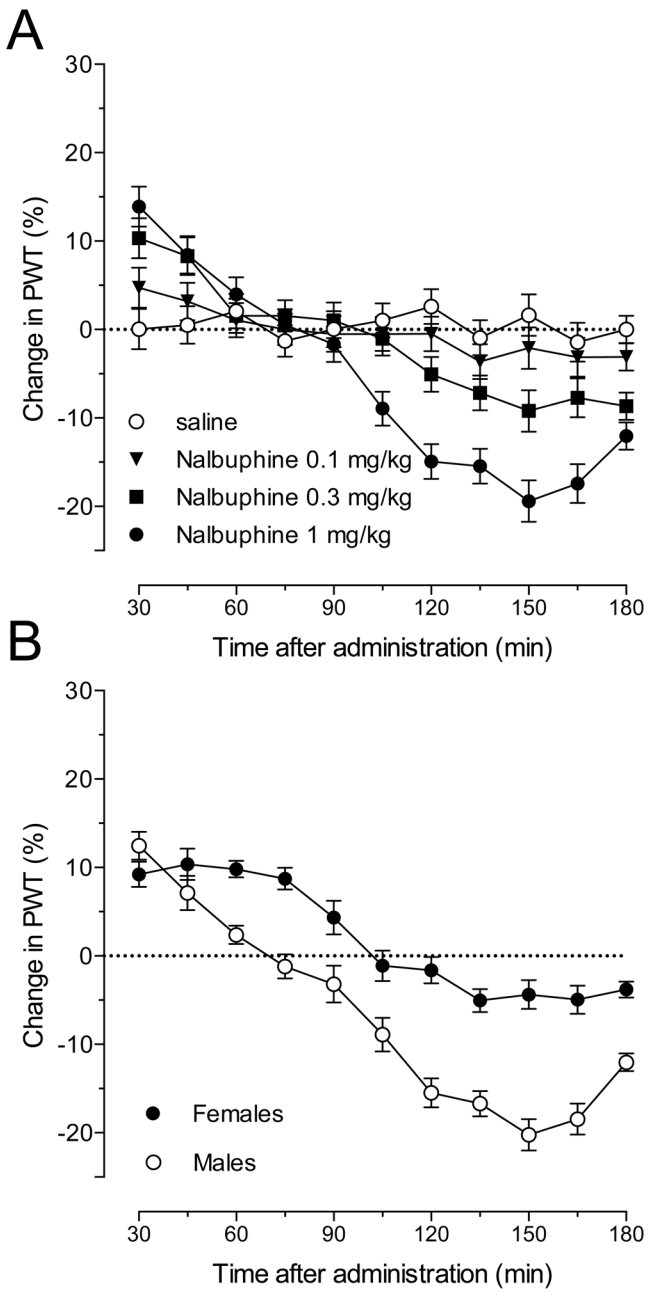

Figure 1. Nalbuphine in the rat

A. Groups of male rats were administered nalbuphine (0.1, 0.3, and 1.0 mg/kg i.v.) or saline (i.v.). Nalbuphine (1 mg/kg) produced both analgesia and anti-analgesia. The onset of anti-analgesia began ~90 minutes after its administration, became significant at 120 minutes, and persisted through the remainder of the 180 minute experiment. (Absolute baseline thresholds: 0.1 mg/kg: 107.7±1.5 g; 0.3 mg/kg: 107.3±0.7 g; 1 mg/kg: 111.6±0.7 g).

B. In female rats, nalbuphine 1 mg/kg produced only analgesia. Data for males is replotted from Fig. 1A. (Absolute baseline threshold for females: 102.2±1.0 g).

In this and subsequent figures, data are plotted as mean ± SEM; analgesia is defined as mechanical nociceptive threshold significantly above baseline; anti-analgesia is defined as threshold significantly below baseline.

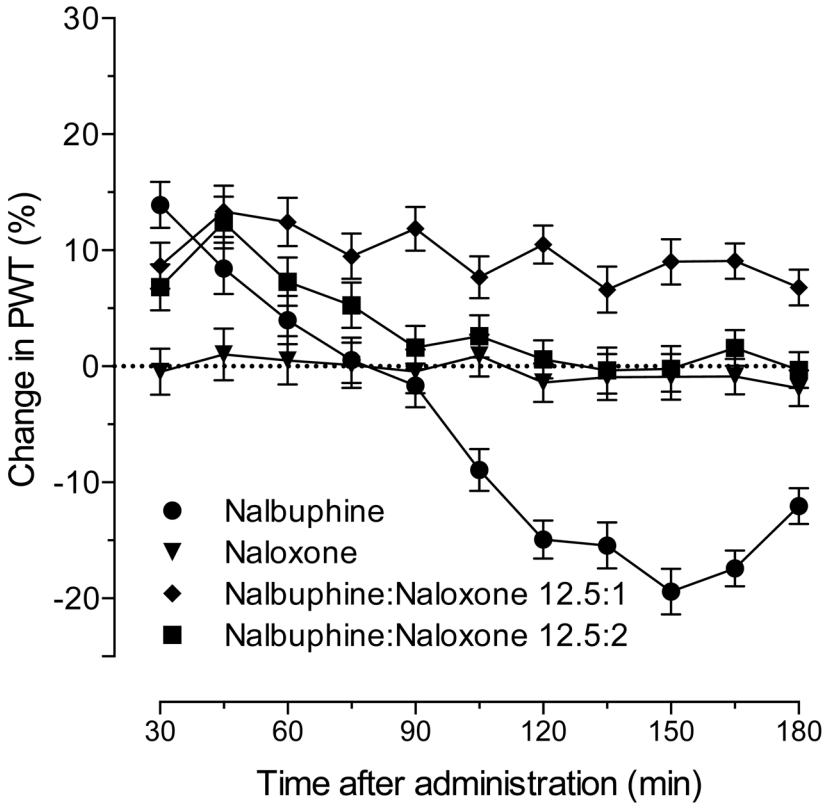

Figure 2. Naloxone co-administered with nalbuphine

Naloxone (80 or 160 μg/kg, i.v.) co-administered with nalbuphine (1 mg/kg, i.v.) blocked anti-analgesia. At the lower dose of naloxone (nalbuphine:naloxone 12.5:1) nalbuphine produced greater analgesia than when administered with the higher dose of naloxone (nalbuphine:naloxone 12.5:2). Naloxone alone (naloxone alone, 80 μg/kg, i.v.) did not affect nociceptive threshold. In this and subsequent figures the data for the nalbuphine (1 mg) alone group is replotted from Figure 1 for comparison purposes only. (Absolute baseline thresholds: 12.5:1 ratio: 117.7±1.7 g; 12.5:2 ratio: 109.9±2.2 g).

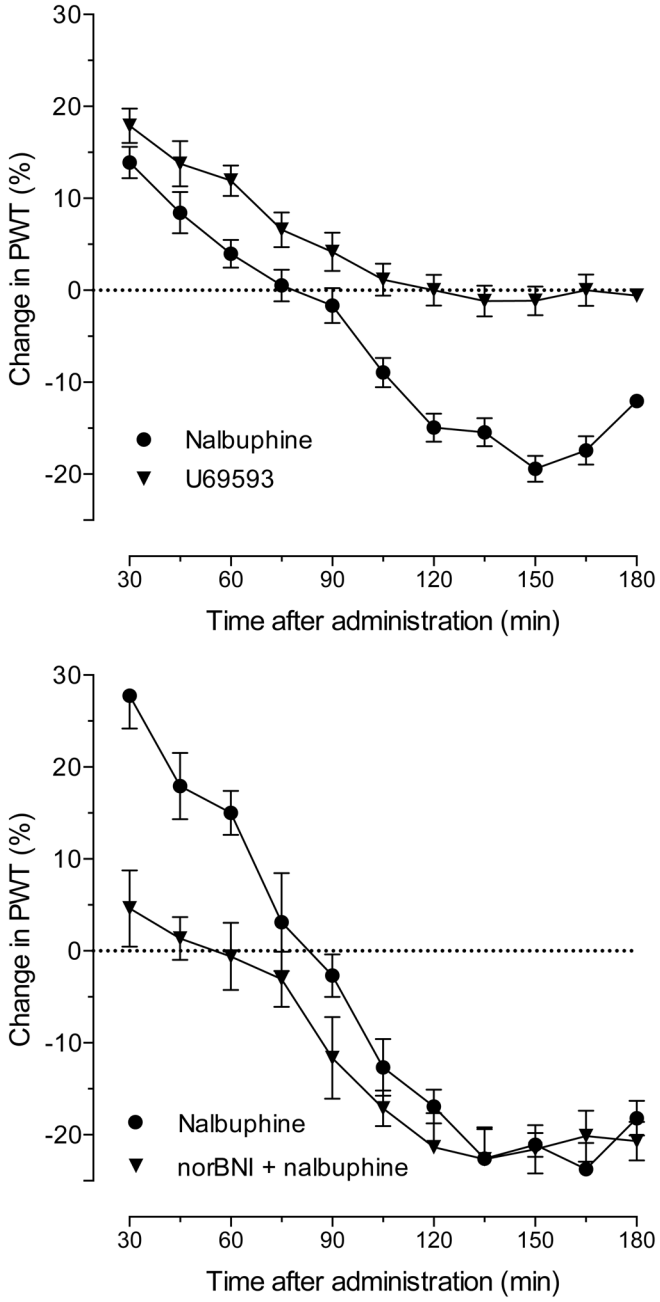

Figure 3.

A. U69,593. The selective κ-opioid receptor agonist U69,593 (0.3 mg/kg, i.v.) produced analgesia of similar magnitude to nalbuphine but not anti-analgesia, supporting the suggestion that nalbuphine anti-analgesia is not induced by its action at κ-receptors. (Absolute threshold: 111.6±0.65 g). Data for the nalbuphine (1 mg) alone group is replotted from Figure 1 for comparison purposes.

B. norBNI. The κ-opioid receptor antagonist norBNI (10 mg/kg, s.c.) given 24 h before nalbuphine (1 mg/kg, i.v.) attenuated its analgesia, but did not affect its anti-analgesia, supporting the suggestion that nalbuphine anti-analgesia is not induced by its action at κ-receptors. (Absolute threshold: 118.7±2.0 g).

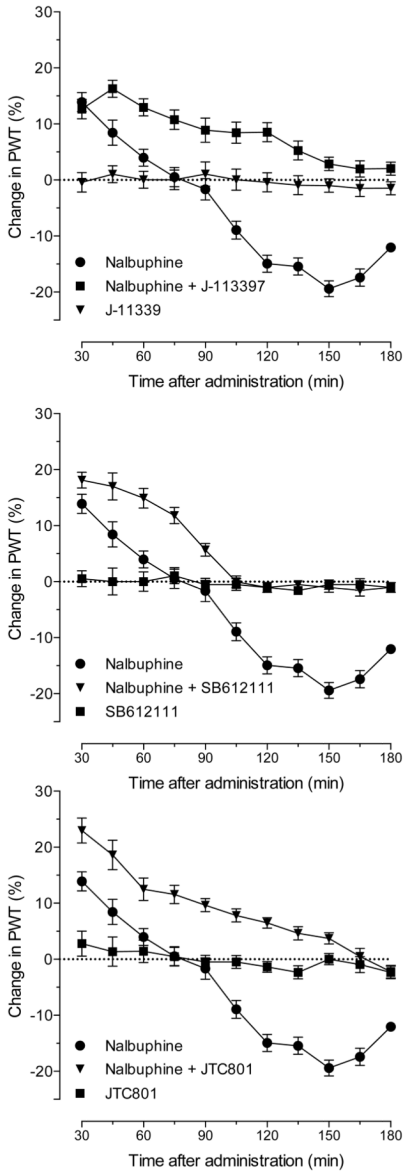

Figure 4. NOP receptor antagonists

Selective NOP-receptor antagonists were administered in separate groups of rats 45 minutes prior to nalbuphine (1 mg/kg, i.v.) or saline (i.v.). In the presence of the antagonist nalbuphine only produced analgesia, indicating the selective blockade of anti-analgesia. There were no significant changes in nociceptive threshold to any of the NOP receptor antagonists when they were administered with saline. A. J-113397; B. SB-612111; C. JTC801. (Absolute baseline thresholds: SB-612111: 107.7±0.7 g; SB-612111+Nalbuphine: 108.4±0.7 g; JTC alone: 118.8±1.3 g; JTC+Nalbuphine: 121.1±1.3 g). Data for the nalbuphine (1 mg) alone group is replotted from Figure 1 for comparison purposes.

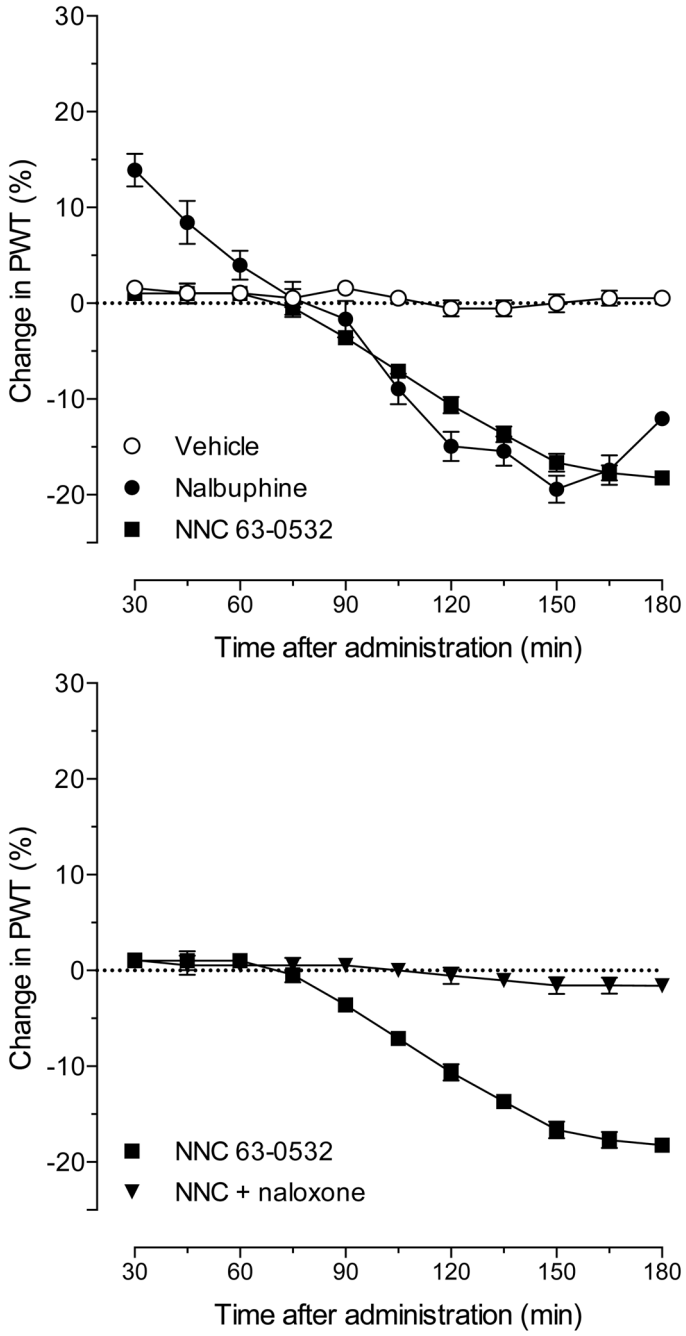

Figure 5. NNC 63-0532

A. The selective NOP-receptor agonist NNC 63-0532 (0.3 mg/kg i.v.) produced no analgesia, but mimicked the anti-analgesic effect of nalbuphine (nalb) starting at 90 minutes and persisting through the remainder of the experiment. Administration of its vehicle did not have a significant effect. (Absolute baseline threshold for NNC 63-0532: 110.0±0.8 g). Data for the nalbuphine (1 mg) alone group is replotted from Figure 1 for comparison purposes. B. Co-administration of naloxone blocked NNC 63-0532-induced anti-analgesia. (Absolute baseline threshold for NNC 63-0532 + naloxone: 105.0±0.6 g).

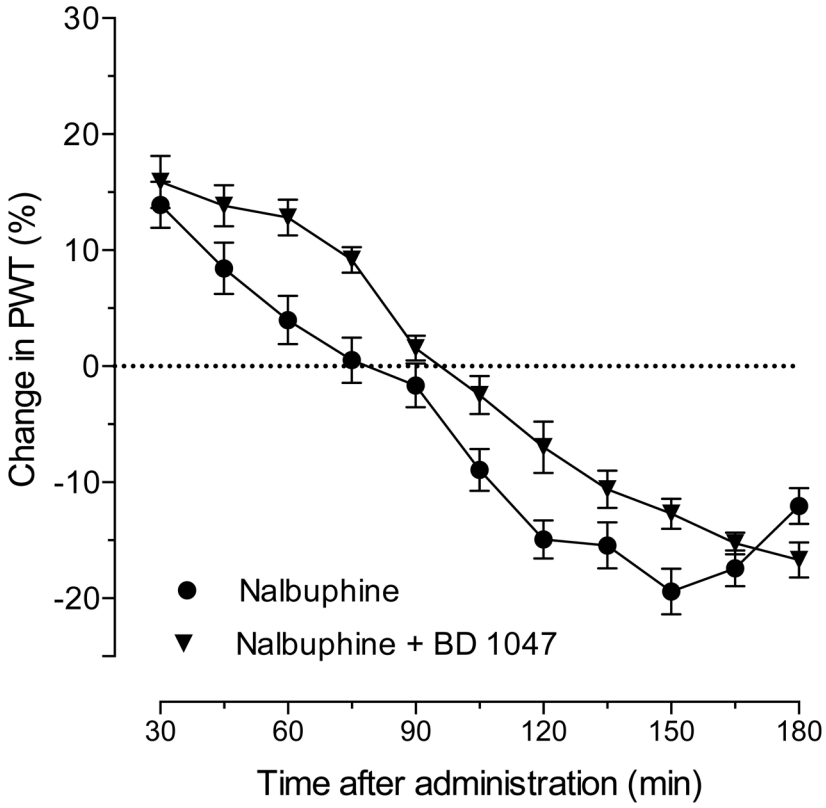

Figure 6. BD 1047

The selective σ-receptor antagonist BD 1047 (10 mg/kg, s.c.) was administered 45 minutes prior to nalbuphine (1 mg/kg, i.v.). Similar to the effect of nalbuphine alone, both analgesia and anti-analgesia were observed. The first four time points demonstrated significant analgesia; the last four time points demonstrated significant anti-analgesia. These results indicate that BD 1047 attenuated neither the analgesic nor the anti-analgesic effects of nalbuphine. (Absolute baseline threshold for BD 1047+nalbuphine: 108.8±1.3 g). Data for the nalbuphine (1 mg) alone group is replotted from Figure 1 for comparison purposes.

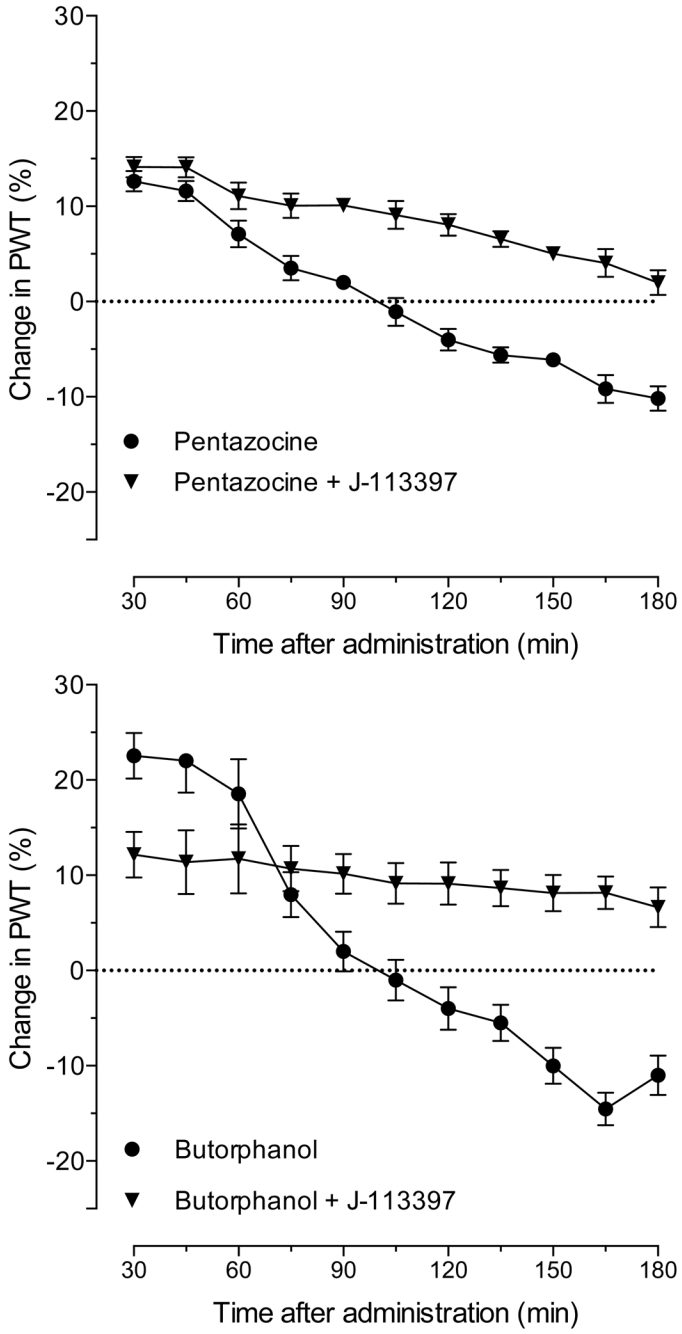

Figure 7. Pentazocine or butorphanol co-administered with J-113397

Pentazocine (5 mg/kg, i.v.), panel A., and butorphanol (0.2 mg/kg, i.v.), panel B., both induced analgesia and delayed onset anti-analgesia; saline vehicle was administered 45 min before pentazocine or butorphanol. In the presence of J-113397 (30 mg/kg, s.c.) administered 45 minutes prior, these κ-agonist-antagonists produced only analgesia, indicating that J-113397 effectively blocked anti-analgesia, similar to its effect when administered with nalbuphine. (Absolute baseline thresholds for pentazocine: 109.4±1.5 g; butorphanol: 111.1±0.6 g; pentazocine+J113397: 110.0±0.8 g; butorphanol+J113397: 108.2±1.7 g)

Table 1

NOP and sigma receptor binding conditions

Receptor	**NOP**	**sigma 1 (σ_1)**	**sigma 2 (σ_2)**
Source	human recombinant HEK-293 cells	human jurkat cells	Wistar Rat brain
Ligand	0.6 nM [^{3}H] nociceptin	8 nM [^{3}H] haloperidol	3 nM [^{3}H] ifenprodil
Vehicle	1% DMSO	1% DMSO	1% DMSO
Incubation time/temp	60 min @ 25.C	4 h @ 25°C	60 min @ 37°C
Incubation buffer	50 mM HEPES, pH 7.4, 1 mM EDTA, 10 mM MgCl, 0.01% bacitracin	5 mM potassium phosphate, pH 7.5	50 mM Tris-HCl, pH 7.4
Non-specific ligand	1 μM orphanin-FQ	10 μM haloperidol	10 μM ifenprodil
K_D	1 nM	5.8 nM	4.8 nM
B_{MAX}	4.6 pmole/mg protein	0.61 pmole/mg protein	1.3 pmole/mg protein
Specific binding	85%	80%	85%
Quantitation method	radioligand binding	radioligand binding	radioligand binding

Binding assay. The experimental conditions for testing nalbuphine, pentazocine, and naloxone binding to NOP, σ_1 or σ_2 receptors.

Table 2

Nalbuphine, naloxone and pentazocine binding to candidate neurotransmitter receptors

Samples of nalbuphine (10 μM), naloxone (10 μM), and pentazocine (10 μM) were tested to determine their ability to inhibit binding of NOP, σ_1, and σ_2 ligand standards to their receptors. Whereas all three drugs demonstrate binding to the NOP receptor, nalbuphine does not bind to the σ_1 receptor and naloxone does not bind to the σ_2 receptor.

	Inhibition (%)		
	Nalbuphine	**Naloxone**	**Pentazocine**
Nociceptin/orphanin F/Q (NOP)	12	19	24
Sigma1 (σ_1)	−2	9	93
Sigma2 (σ_2)	15	−2	81