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Absence of conditioned place preference or reinstatement with bivalent ligands containing mu-opioid receptor agonist and delta-opioid receptor antagonist pharmacophores

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Abstract

Treatment of pain with opioids is limited by their potential abuse liability. In an effort to develop analgesics without this side effect, a series of bivalent ligands containing a mu-opioid receptor agonist pharmacophore connected to a delta-opioid receptor antagonist pharmacophore through variable-length spacers (16–21 atoms) was synthesized. Members of this series [mu-opioid receptor (M)-delta-opioid receptor (D)-agonist (A)-antagonists (N): MDANs] are antinociceptive in the tail flick assay, but antinociceptive tolerance and physical dependence do not develop to ligands having spacers with 19–21 atoms. The current studies compared the rewarding properties of three bivalent ligands (MDAN-16, -19 and -21) and a mu-opioid receptor agonist (MA-19) to those of morphine in the conditioned place preference assay in mice after i.v. administration. Place preference developed to morphine and to MA-19, but not to the MDANs. The responses to MDAN-16 were highly variable, although place preference of borderline significance appeared to develop. Reinstatement was also evaluated after extinguishing morphine conditioned place preference; morphine and MA-19, but not the MDANs, reinstated morphine conditioned place preference. Taken together, these results suggest that the bivalents are less rewarding compared to morphine in opioid-naïve mice and do not induce reinstatement in previously morphine-preferring mice. The lack of a conditioned place preference response for MDAN-19 and -21, compared to the equivocal results with MDAN-16, suggests a minimum distance requirement between mu-opioid receptor and delta-opioid receptor recognition sites. This requirement may reflect the binding of MDAN-19 and -21 to mu-opioid receptor–delta-opioid receptor heterodimeric receptors that block reward but not antinociception. Published by Elsevier B.V.

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1. Introduction

Opioids are the most efficacious medications for the treatment of moderate to severe pain, but chronic treatment leads to development of tolerance and physical dependence that is accompanied by high abuse liability. In an effort to develop opioids devoid of these side effects, we recently described the synthesis of novel opioid compounds comprising a mu-opioid receptor agonist (oxymorphone) linked to a delta-opioid receptor antagonist (naltrindole) by spacers of variable length (19–25 Å) (Daniels et al., 2005). These compounds are designated MDAN-16, -17, -18, -19, -20, and -21, with M=mu, D=delta, A=agonist, N=antagonist, and the number indicating the number of atoms in the spacer. The spacer length was varied to optimize the distance between the two pharmacophores to enhance the binding to putative mu-opioid receptor–delta-opioid receptor heterodimeric receptors (George et al., 2000; Gomes et al., 2000).

The MDAN bivalent ligands are all more potent (after i.c.v. administration) than morphine in the tail flick assay, with the spacer length directly related to potency (i.e., MDAN-16 is the least potent, while MDAN-21 is the most potent) (Daniels et al., 2005). In addition, MDAN-21 is 50-fold more potent than morphine after i.v. administration. Importantly, neither tolerance nor physical dependence develops to the MDAN ligands with the longest spacer length (MDAN-19, -20, -21) after continuous

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infusion i.c.v. for 3 d, a protocol in which tolerance and physical dependence develop to morphine (Lenard and Roerig, 2005). It should also be noted that tolerance, but not physical dependence, develops to MDAN ligands with shorter spacers (MDAN-17 and -18) while both tolerance and physical dependence developed to the MDAN ligand with the shortest spacer (MDAN-16). Other experiments showed that mice infused i.c.v. for 3 d with MDAN-20 were not cross-tolerant to morphine, whereas mice infused for 3 d with morphine were cross-tolerant to MDAN-21 (unpublished observations). Finally, when the MDAN ligands were administered i.v., minimal inhibition of gastrointestinal transit was observed, whereas morphine effectively inhibited gastrointestinal transit with an ED50 value lower than the tail flick antinociceptive ED50 value (Lenard et al., 2005).

Because addiction liability limits the clinical use of opioids, experiments were designed to test the effects of the MDAN compounds in a model of addiction liability. Many drugs (e.g., heroin, cocaine, etc.) are often abused for their rewarding properties, i.e., to get "high," which can lead to addiction. The conditioned place preference paradigm is often used as an animal model to predict whether a drug is likely to be rewarding and/or aversive to humans. The majority of drugs abused by humans will also promote conditioned place preference in rodents (Tzschentke, 1998). The first aim of the present studies was to determine whether the MDANs, like morphine, support conditioned place preference in mice. A lack of conditioned place preference induced by the MDANs would support their attractiveness as clinically useful analgesics without abuse liability.

Drug-seeking behavior, like that seen in the conditioned place preference paradigm or in self-administration studies, is terminated in a variety of ways. This process, termed extinction, thus models the abstinence phase of human drug rehabilitation. Once the behavior is extinguished, noncontingent injections of the drug can reinstate the drug-seeking behavior in a model of relapse (Shaham et al., 2003). Other drugs, both within (e.g., heroin vs. morphine) and outside (e.g., heroin vs. cocaine) the drug class can similarly reinstate drug-seeking behavior (Shalev et al., 2002; Lu et al., 2004). Thus, a second aim of the current studies was to determine whether noncontingent injections of the MDANs reinstate previously extinguished morphine-induced conditioned place preference.

In the current studies, the abuse potential of newly synthesized, potent opioid analgesics was assessed using the conditioned place preference paradigm in mice. First, mice were conditioned with the MDANs themselves to determine whether conditioned place preference was induced. Second, the abilities of the MDANs to reinstate previously extinguished morphine-induced conditioned place preference were evaluated. Knowing these characteristics will likely be useful for the future development of the MDANs as analgesic medications.

2. Methods

2.1. Animals

Male ICR mice (Harlan, Indianapolis, IN) that weighed 28–45 g were used throughout these studies. Although we recognize the importance of strain in conditioned place preference response (Semenova et al., 1995), we chose to use ICR (Xu et al., 2001; Grabus et al., 2006) mice to be consistent with our previous studies investigating the development of tolerance and physical dependence to these ligands (Daniels et al., 2005). The animals were housed in groups of 5 at an ambient temperature of 22–23 °C in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited animal care facility under a 12–12 h light/dark cycle. Both food and water were available ad libitum, except during behavioral testing. All procedures complied with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and were approved by the LSUHSC-S Animal Care and Use Committee. Each mouse was used only once for the tail flick experiments or in the acquisition or reinstatement of conditioned place preference paradigms.

2.2. Drug administration

Drugs were dissolved in sterile saline (0.9% NaCl) and administered with a 1-ml syringe and a 27-g needle in a volume of 100 μl into a lateral tail vein. Drugs were administered by hand, by one investigator, with care taken that the rate of drug infusion was as uniform as possible. Although most conditioned place preference studies use i.p. or s.c. administration, i.v. administration was employed for all experiments to conserve the limited supply of bivalent ligands.

2.3. Antinociceptive testing

Antinociception was evaluated by the radiant heat tail flick assay (D'Amour and Smith, 1941). Briefly, a beam of light was focused on the tail and the time until the tail flicked was measured. The light intensity was adjusted so that control times were between 1.5 and 2.5 s. A 10-s cutoff drug time was set to minimize the risk of tissue damage. Each animal served as its own control and was used only once. Mice were tested once before injection (control time) and again 15 min after drug administration (drug time). Percent maximum possible effect (% MPE) was calculated as follows (Dewey et al., 1970):

$$\frac{\text{Drug Time (s)-Control Time (s)}}{\text{10 s-Control Time (s)}} \times 100\% = \%\ \text{MPE}$$

Graded dose response curves of at least 4 doses with at least (8–10) mice per dose were generated from the % MPE data. ED50 values with 95% confidence intervals were computed with GraphPad Prism using nonlinear regression methods.

2.4. Conditioned place preference

2.4.1. Apparatus

One plastic box with two equal size compartments (13.5 cm wide × 16 cm deep × 16 cm high) was used. The compartments were either clear with a scored floor or blue striped with a smooth floor. The halves were separated with a divider

containing an opening (6.5 cm wide × 6.5 cm high) or a divider without an opening.

2.4.2. Acquisition of place preference

The place conditioning procedure consisted of four phases: exposure to a novel environment, preconditioning, conditioning, and postconditioning. In the first phase (exposure to a novel environment), the mice were allowed to explore both sides of the apparatus for 15 min (Day 1). In the second phase (preconditioning), the time the mice spent in each side of the box out of a total of 15 min was recorded (Day 2). The first placement of the animals into the box was randomized to the drug-paired side or the saline-paired side. Animals were considered to have crossed to the other side when all four paws were in that compartment. Animals that showed a strong initial preference or aversion (i.e., <180 s or >540 s) for a particular side were excluded from the studies (~10%).

In the third phase (conditioning, Days 3–5), the mice were injected with saline and confined to one half of the box for 30 min. Half of the mice were randomized to be conditioned with saline on one side of the box and half were conditioned on the other side. The same day, after the saline injections were completed, the mice were injected with drugs and confined to the side of the box that was not previously paired with saline. This conditioning regimen continued for a total of 3 d so that the animals were paired with saline 3 times and the drug 3 times. In this manner all animals were conditioned with both saline and the experimental drug.

We chose morphine as our positive control rather than oxymorphone, the parent compound for two reasons. One, there is an extensive literature for morphine conditioned place preference in rodents (see review by Tzschentke, 1998) and two, to verify that the conditions used in our protocol would, indeed, produce conditioned place preference. Preliminary experiments with morphine were completed with a dose 2× the ED90 dose for antinociception (Fig. 2). Because there was no place preference to this dose, subsequent experiments with morphine were completed with a dose 4× the ED90 dose for antinociception (Fig. 2). Whereas four times the ED90 dose for antinociception (1200 nmol) is somewhat high when compared to the ~500 nmol (i.v.) dose used for conditioning in rats (Mucha and Walker, 1987), it is likely that species differences account for the higher dose of morphine needed for conditioning in mice. Indeed, a review of the literature suggests that higher doses of morphine are used for conditioned place preference studies in mice compared those in rats (Bardo and Neisewander, 1986; Manzanedo et al., 2001; Frances et al., 2004; Fenu et al., 2006). This dose (i.e., 4× the ED90 for antinociception) was used for all the remaining drugs. No behavioral or locomotor stimulation effects were observed with this dose. We did not, however, quantitate the number of times each mouse crossed from one compartment to the other. In experiments with antagonists, the antagonist was coadministered with the agonist. Naloxone was administered at a dose approximately 25% of the agonist it was being coadministered with. The delta-selective opioid receptor antagonist was coadministered with the mu-selective opioid agonist at equimolar doses.

In the final phase (postconditioning), the animals were again allowed to explore both sides of the box for 15 min. These conditioning and testing conditions are similar to those reported by another laboratory (Olson et al., 2006, supplemental material). The time the mice spent in each side of the box was again recorded, just as in the preconditioning phase (Day 6). It should be noted that the observer was not "blinded" regarding which side of the box was paired with drug and which was paired with saline. The time in seconds spent in the drug-paired side in the postconditioning phase was subtracted from the time spent in the drug-paired side in the preconditioning phase. This result, also called the place preference score, was divided by 900 (i.e., 15 min) and multiplied by 100%. The dependent variable was the % change in time spent in the drug-paired side. If this number is positive, then the drug has induced place preference, and if it is negative, the drug has induced place aversion.

2.4.3. Extinction of place preference

Mice were conditioned with morphine and tested for place preference as described. The mice were then returned to their home cages until the following day. The mice were allowed to explore both sides of the apparatus for 15 min once daily to extinguish the place preference. The place preference was considered extinguished when it was less than 20% of the initial preference. In general, the place preference was still evident on the day after the initial place preference testing, but was absent on the next day. Thus, two days of extinction was usually required to extinguish the morphine-induced place preference, with a maximum of three days.

2.4.4. Reinstatement of place preference

On the day following extinction, the mice were injected with the ED90 antinociceptive dose (priming injection) of the drug to be tested for its ability to reinstate previously extinguished morphine-induced place preference. This dose, then, was 25% of the conditioning dose and was in the antinociceptive range. Antagonists were coadministered with the agonists. Place preference testing took place as described previously. Separate groups of mice underwent conditioning with morphine, extinction of the preference, reinstatement for each priming drug tested.

2.5. Drugs

Morphine sulfate was obtained from NIDA (Baltimore, MD). Naloxone hydrochloride was obtained from Sigma (St. Louis, MO). The novel compounds were synthesized according to Daniels et al. (2005). MA-19 is the mu-opioid receptor agonist oxymorphone with an attached spacer of 19 atoms (22.9 Å), DN-20 is the delta-opioid antagonist naltrindole with an attached spacer of 20 atoms (24.1 Å), and the MDAN compounds are oxymorphone attached to naltrindole with spacers of 16, 19 or 21 atoms (19.1, 22.9, 25.4 Å). The base was used in calculating doses of the drug.

2.6. Statistical analyses

Data are presented as means ± S.E.M. ED50 values were considered significantly different when the 95% confidence intervals

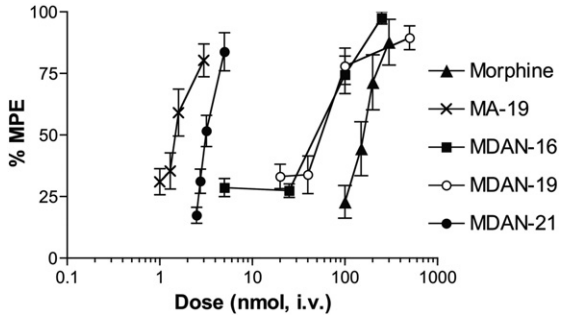

Fig. 1. Acute antinociceptive effects of morphine, the monovalent mu-opioid receptor agonist MA-19 and the bivalent mu-opioid receptor agonist–delta-opioid receptor antagonist compounds MDAN-16, -19, and -21 in the tail flick test. Drugs were injected i.v. and antinociception was measured 15 min later. Each point represents the mean of 4–14 mice±S.E.M.

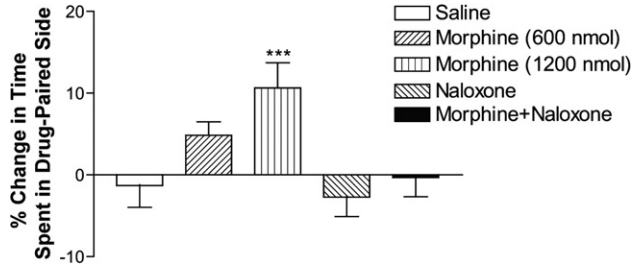

Fig. 2. Morphine-induced conditioned place preference. Mice (n=5–8) were conditioned with saline, morphine (600 or 1200 nmol), naloxone (300 nmol), or morphine (1200 nmol) plus naloxone (300 nmol) for three days and tested for place preference on the fourth day. *Significantly different from saline, one-way ANOVA followed by the Dunnett test, $P<0.05$.

did not overlap. Conditioned place preference scores were presented as % change in time spent in drug-paired side to facilitate comparison of figures. For 2-group comparisons (i.e., fentanyl vs. saline), Student's 2-tailed *t*-test was used. For more than 2-group comparisons, one-way ANOVA followed by the Dunnett multiple comparison test was used to compare all groups to saline. It should be noted that similar significance values were obtained when the data were represented as conditioned place preference score (rather than % change) and tested with Newman–Keuls multiple comparison test (rather than Dunnett's). In the reinstatement studies, the data were compared using a one-way ANOVA followed by the Newman–Keuls multiple comparison test to compare all 3 groups to each other. Significance was accepted at $P<0.05$.

3. Results

3.1. Antinociceptive effects of acutely administered drugs

To determine the antinociceptive effects of the drugs after i.v. administration, morphine, the monovalent mu-opioid receptor agonist MA-19, and the bivalent mu-opioid receptor agonist-delta-opioid receptor antagonist compounds MDAN-16, -19, and -21 were administered i.v. 15 min before testing in the tail flick assay (Fig. 1). All drugs were fully efficacious in the tail flick assay. Morphine was the least potent [ED50 (95% CI)=168 (146–178) nmol] and MA-19 was the most potent [ED50=1.6 (1.4–1.7) nmol]. This relationship was similar to that found after i.c.v. administration of these agents (Daniels et al., 2005). Of the bivalent ligands, MDAN-16 [ED50=45 (7.1–82) nmol] and MDAN-19 [ED50=36 (22–50) nmol] were equipotent, whereas MDAN-21 was the most potent [ED50=3.3 (3.0–3.6 nmol)]. Again, these findings are similar overall to the potency relationship found after i.c.v. administration, except that after i.c.v. administration, MDAN-19 was more potent than MDAN-16.

3.2. Conditioned place preference induced by morphine

Mice were conditioned with two doses of morphine for three days (Fig. 2). With the lower dose (600 nmol/d; 2× the ED90 for antinociception), the place preference was positive but not different from that of saline. With the higher dose of morphine (1200 nmol/d; 4× the ED90 dose for antinociception), the place preference score was both positive and different from that of saline. The mu-selective opioid receptor agonist fentanyl (8 nmol/d; 4× the ED90 dose for antinociception) induced a similar place preference (8.2% vs. 10.6% for morphine, data not shown). This % change was different from that of saline ($P<0.05$, Student's *t*-test).

When mice were conditioned with 1200 nmol of morphine plus 300 nmol (i.e., 25% of the conditioning dose of the agonist) of the nonselective opioid receptor antagonist naloxone, place preference was not observed. Thus, as expected, the antagonist blocked the effect of the agonist. Mice were also conditioned with naloxone (300 nmol) alone. The resulting place preference score for naloxone was not different from that observed for saline-conditioned animals (Fig. 2), indicating that treatment with the antagonist alone did not produce place preference or aversion. Similarly, others have shown that naloxone does not produce conditioned place preference in ICR mice (Kim et al, 1997). Thus, it appears naloxone blocks the effects of morphine, instead of counteracting the rewarding effects of morphine by producing aversive effects of its own.

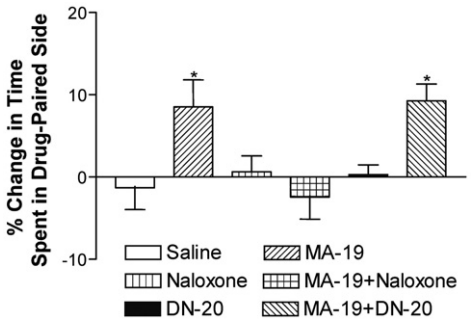

Fig. 3. Conditioned place preference induced by the monovalent mu-receptor agonist MA-19. Mice (n=6–8) were conditioned with saline (same mice as in Fig. 2), MA-19 (12 nmol), naloxone (3 nmol), MA-19 (12 nmol) plus naloxone (3 nmol), the delta-opioid receptor antagonist DN-20 (12 nmol), or MA-19 (12 nmol) plus DN-20 (12 nmol) for three days and tested for place preference on the fourth day. *Significantly different from saline, one-way ANOVA followed by the Dunnett test, $P<0.05$.

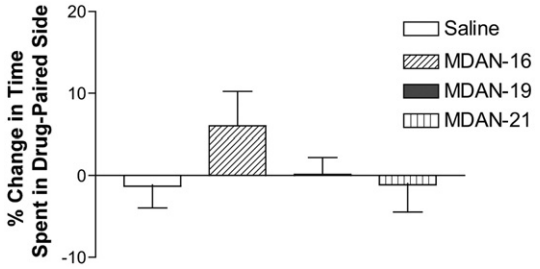

Fig. 4. Conditioned place preference induced by the mu-opioid receptor agonist–delta-opioid receptor antagonist bivalent compounds. Mice (6–10) were conditioned with saline (same mice as in Figs. 2 and 3), MDAN-16 (800 nmol), MDAN-19 (800 nmol), or MDAN-21 (20 nmol) for three days and tested for place preference on the fourth day.

3.3. Conditioned place preference induced by the monovalent and bivalent opioids

The mice were conditioned with monovalent and bivalent ligands at a dose of 4× the ED90 dose for antinociception (Figs. 3 and 4). The monovalent mu-opioid receptor agonist MA-19 (12 nmol) induced a place preference that was similar to that of morphine. This preference was not seen when mice were conditioned with MA-19 coadministered with naloxone (3 nmol, Fig. 3, 25% of the conditioning dose of the agonist). Thus, as found for morphine, the nonselective antagonist blocked the effect of the mu-opioid receptor agonist.

In a separate experiment, the animals were conditioned with MA-19 (12 nmol) in combination with the delta-opioid receptor antagonist DN-20 (12 nmol) and the resulting place preference was similar to that of MA-19 alone (Fig. 3). It should be noted that the antagonist dose was equal to the agonist dose, rather than 25% of the agonist dose, as was the case with naloxone and morphine. We chose this paradigm to compare the administration of two monovalent drugs (i.e., MA-19 and DN-20) with the administration of the bivalent drug, as seen with the MDANs (i.e., MDAN-16, -19, and -21). Neither place preference nor aversion was induced by this dose of DN-20. These results suggest that the delta-opioid receptor antagonist does not mitigate the rewarding effects of MA-19 when the two are not linked together. This finding agrees with the previous report showing that DN-20 does not attenuate the antinociceptive effect of MA-19 (Daniels et al., 2005).

The next set of experiments was designed to determine the effects of the mu-opioid receptor agonist linked to the delta-opioid receptor antagonist for producing conditioned place preference. Of the bivalent ligands tested, MDAN-16 showed a tendency to induce a positive place preference, but results were not different from those observed for saline. Of the 10 mice tested, 4 displayed conditioned place preference scores greater than 10% over preconditioning score indicating place preference, 3 displayed a place aversion less than 10% of the preconditioning score, and 3 displayed conditioned place preference scores less than 10% different of the preconditioning score. These results demonstrated a wide variation in responses to MDAN-16.

The percent change in preference during conditioning with MDAN-19 was very small and not different from that of saline, indicating a lack of conditioned place preference. Similar results were obtained when MDAN-21 was tested (Fig. 4).

3.4. Reinstatement of morphine-induced place preference with priming injections of morphine

Mice were conditioned with morphine for three days, tested for place preference, and then the place preference was extinguished as described in Methods. On the next day, mice were tested with the challenge drugs using the same doses previously used for determination of conditioned place preference (above). As shown in Fig. 5, saline did not induce reinstatement of morphine conditioned place preference. As expected, a morphine injection induced a return of the previously extinguished place preference. This preference appeared to be somewhat greater than the initial preference, but the difference was not significant.

A priming injection of MA-19 (3 nmol) induced a return of morphine-induced initial place preference that was even more robust than the initial place preference (Fig. 5). Similar results were observed with a priming injection of MA-19 (3 nmol) coadministered with DN-20 (3 nmol), except that the reinstatement place preference was not different than the initial place preference. Thus, similar to what was seen in the acquisition

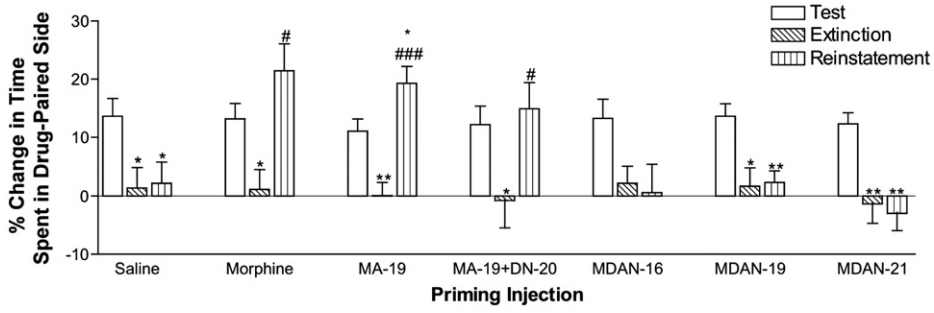

Fig. 5. Priming injection-induced reinstatement of morphine conditioned place preference. Mice (n=5–8) were conditioned with morphine and tested (Test). The morphine place preference was extinguished during daily extinction sessions. On the day the preference was <20% of the preference, the place preference was considered to be extinguished (Extinction). The next day, the mice were injected with various drugs (Priming Injection) to determine whether they reinstated the morphine place preference. *Significantly different from Test, one-way ANOVA followed by the Newman–Keuls test $P<0.05$, **$P<0.01$. $^{\#}$Significantly different from Extinction, one-way ANOVA followed by the Newman–Keuls test, $P<0.05$, $^{\#\#\#}P<0.001$.

experiments, DN-20 did not block the effects of MA-19 on reinstatement.

None of the bivalent ligands [MDAN-16 (200 nmol), MDAN-19 (200 nmol); MDAN-21 (200 nmol)] induced a return of the previously extinguished morphine place preference. These results suggest that the bivalent ligands would not induce relapse in patients previously addicted to morphine.

4. Discussion

Delta-opioid receptor antagonists prevent or attenuate the development of tolerance and dependence to morphine (Abdelhamid et al., 1991; Hepburn et al., 1997; Fundytus et al., 1995). Zhu et al. (1999) showed that tolerance does not develop in mice lacking the delta-opioid receptor. In our recent report, bivalent ligands consisting of a mu-opioid receptor agonist coupled to a delta-opioid receptor antagonist by variable-length spacers were synthesized as potent analgesics with decreased tolerance and dependence liability. The ligands with spacer lengths of 23–25 Å did not result in antinociceptive tolerance or physical dependence (Daniels et al, 2005). The current studies compare the acquisition of conditioned place preference in the same series of ligands (MDANs) to a mu monovalent receptor agonist and to morphine. In addition, the reinstatement of morphine-induced place preference by noncontingent priming injections of these drugs was examined. Both morphine and the mu monovalent ligand (MA-19) induced place preference and reinstatement of morphine-induced place preference, whereas none of the MDAN bivalent ligands did, although equivocal results were obtained with MDAN-16 for place preference.

Careful examination of the response patterns suggests that conditioned place preference develops in parallel with tolerance and physical dependence; i.e., we found that larger conditioned place preference scores are obtained with drugs that result in the most tolerance and physical dependence. Mice infused i.c.v. for 3 days with morphine or MA-19 develop considerable tolerance and dependence, as shown by increased antinociceptive ED50 values and robust naloxone-induced jumping (Daniels et al., 2005). However, after infusion with either MDAN-19 or MDAN-21, neither tolerance nor dependence is observed. Interestingly, after MDAN-16 infusion, mice show an approximate 3-fold increase in antinociceptive ED50 values (compared to 6-fold with morphine), as well as jumping (~1/3 of that with morphine). In the present studies, morphine and MA-19 induced conditioned place preference, whereas MDAN-19 and -21 did not. Notably, although conditioned place preference induced by MDAN-16 was highly variable, the effect was different from the complete absence of conditioned place preference shown for MDAN-19 and -21. Thus, the development of tolerance, dependence and conditioned place preference to MDAN-16 was intermediate between the mu ligands (morphine and MA-19) and the bivalent mu-opioid receptor agonist–delta-opioid receptor antagonist ligands.

The high variability of results for MDAN-16 could be due to the shorter spacer allowing binding of MDAN-16 either to mainly mu-opioid receptors or to mainly delta-opioid receptors because of suboptimal bridging between the two pharmacophores. Thus, MDAN-16 would be rewarding if it interacted mainly with mu-opioid receptors, with suboptimal binding to delta-opioid receptors (40% of mice tested showed place preference). Interaction mainly with delta-opioid receptors may have resulted in no response to MDAN-16 (30% of mice tested showed neither aversion nor preference) because the DN-20 parent compound, naltrindole, produces neither place preference nor aversion (Ide et al., 2004; Menkens et al., 1992). Also, the delta-opioid receptor antagonist ICI,174864 has no effect in a conditioned place preference paradigm (Shippenberg et al., 1987).

The lack of place preference to MDAN-19 and MDAN-21 was clearly obvious. Because this result is different from that obtained by morphine or MA-19, it is suggested that the bivalent ligands with 22 and 25 Å spacer lengths bind mu-opioid receptor–delta-opioid receptor heterodimers. The role of possible heterodimeric association of mu- and delta-opioid receptors in the development of conditioned place preference is supported by the apparent minimum distance requirement between the two pharmacophores to prevent the acquisition of conditioned place preference; i.e., MDAN-19 and -21 produced essentially no conditioned place preference, whereas equivocal results were seen with MDAN-16. Mu-opioid receptor–delta-opioid receptor heterodimers may use different G proteins and/or signaling mechanisms than do the mu- or delta-opioid receptors acting alone (George et al., 2000; Gomes et al., 2000). It may be that mu-opioid receptor–delta-opioid receptor heterodimers, but not individual mu-opioid receptors, mediate the acquisition of conditioned place preference via intracellular processes connected only to the heterodimers. If this is the case, the negative modulation of these processes via the delta-opioid receptor through optimal bridging of the mu-opioid receptor–delta-opioid receptor heterodimer should reduce the acquisition of conditioned place preference and possibly reward. Expression of conditioned place preference was not related to the antinociceptive effects of the drugs. The three MDAN ligands show fully efficacious antinociceptive activity in the tail flick test that is intermediate in potency between morphine and MA-19, with morphine the least potent and MA-19 the most potent. Thus, mu ligands with the most and least antinociceptive potency, both produced conditioned place preference, whereas the agonist–antagonist ligands with intermediate antinociceptive potency did not produce significant conditioned place preference. Others have previously noted the lack of correlation between antinociceptive potency and rewarding properties in mice (Pchelintsev et al, 1991; Semenova et al., 1995) suggesting that central processes involved in rewarding processes may be dissociated from processes responsible for the expression of antinociception. Given that we based our conditioning doses on antinociceptive ED90 values, the lack of correlation between conditioned place preference and antinociception may be a potential confound in our studies. However, if this were the case we would expect a direct, rather than inverse, relationship between conditioning doses and place preference, and as such, would expect 4× the ED90 value for antinociception to produce conditioned place preference if the drug were reinforcing (and not aversive) in mice.

The acquisition of conditioned place preference to morphine and MA-19 was blocked by coadministration with the non-selective opioid antagonist naloxone. These results for morphine are in agreement with the findings of others (Bilsky et al., 1990; Sora et al., 2001. The acquisition of place preference to MA-19 was not blocked with equimolar doses of the delta-opioid receptor antagonist DN-20. Delta-opioid receptor antagonists have been shown to block (Suzuki et al., 1994), not block (Piepponen et al., 1997), or have no effect on (Shippenberg et al., 1987) morphine-induced place preference. Thus, whether or not the lack of effect of the delta-opioid receptor antagonist on MA-19-induced place preference is expected is debatable. The mu-opioid receptor is thought to mediate the rewarding effects of morphine, as demonstrated by using mu-opioid receptor knock-out mice in the conditioned place preference assay (Matthes et al., 1996). However, Suzuki et al. (1997) demonstrated that antisense oligonucleotides to the delta-opioid receptor decreased the place preference induced by morphine. The current finding, that DN-20 did not affect MA-19 conditioned place preference, agrees with our previous report (Daniels et al., 2005) showing that DN-20 does not block the acute antinociceptive effect of MA-19 or the development of tolerance and physical dependence to chronic MA-19. These findings may appear to contradict the results obtained with the MDAN compounds, but both results could be due to the relatively lower effective local concentration of the antagonist (DN-20) at the receptor when compared to that of the delta-opioid receptor antagonist pharmacophore in the bivalent ligand. This is a consequence of tethering the delta pharmacophore to the mu pharmacophore thereby allowing it to possess greater residence time in the vicinity of the neighboring delta-opioid receptor recognition site. The local concentration of monovalent ligands is lower because they are under no such constraint. Consequently, it appears that the tethering of the delta-opioid receptor antagonist to the mu-opioid receptor agonist is essential for decreasing the acquisition of conditioned place preference and development of tolerance and physical dependence, as is seen with MDAN-19 and -21, and this is consistent with the existence of mu-opioid receptor–delta-opioid receptor heterodimers in the mediation of these effects. A second focus of the current studies was to study the reinstatement of morphine place preference induced by priming injections of the various drugs. According to Mueller et al. (2002), priming injections "remind" the animals of the significance of the contextual stimuli and in turn reinstate drug-seeking behavior. In order to study reinstatement, the place preference must first be extinguished. Extinction of the morphine preference took place after only 2–3 days, perhaps because the initial place preference was not terribly robust. Others have demonstrated that morphine place preference is extinguished in approximately 1 week in mice (Ribeiro Do Couto et al., 2003) but can persist from 7 days (Wang et al., 2000) to 12 weeks in rats (Mueller et al., 2002), depending on the experimental protocol.

Morphine, at antinociceptive rather than conditioning doses, completely reinstated the extinguished place preference to morphine, whereas MA-19 resulted in conditioned place preference greater than that seen with the original conditioning with morphine. Similar enhancements of place preference have been previously reported with reinstatement with morphine in mice (Ribeiro Do Couto et al., 2003). It is likely that sensitization of the reward circuitry in the brain is responsible for this effect. The coadministration of MA-19 and DN-20 also reinstated the morphine place preference. However, the reinstatement was not different from the initial place preference, in contrast to the reinstatement induced by MA-19 alone. These results might imply that the delta-opioid receptor is involved in the sensitization of brain reward circuitry, as suggested by studies of cocaine sensitization (Shippenberg and Heidbreder, 1995).

The reinstatement studies may be actually more compelling than the acquisition studies regarding the abuse liability of the bivalent ligands. The mice conditioned with morphine are already "primed," so to speak, to find drugs rewarding. However, antinociceptive doses of the bivalent ligands did not reinstate the morphine place preference. Again, the results with MDAN-16 did not reach statistical significance, suggesting suboptimal binding of this bivalent ligand to the mu-opioid receptor–delta-opioid receptor heterodimer. The lack of reinstatement seen with the MDANs might be related to dissimilar internal cues generated by the bivalent ligands compared to those generated by morphine. It would be interesting to examine whether the bivalent ligands produce morphine-appropriate responding in discriminative stimulus experiments.

In summary, we have demonstrated that i.v. administration of 3 MDANs did not induce conditioned place preference, whereas the mu monovalent ligand and morphine did. Furthermore, although priming injections of MA-19 or morphine reinstated previously established and extinguished morphine conditioned place preference, none of the MDANs had that effect. These data suggest that the MDAN bivalent ligands may be effective drugs, with reduced abuse liability, for the treatment of pain in both opioid-naïve and formerly addicted patients.

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References

Abdelhamid, E.E., Sultana, M., Portoghese, P.S., Takemori, A.E., 1991. Selective blockage of delta opioid receptors prevents the development of morphine tolerance and dependence in mice. J. Pharmacol. Exp. Ther. 258, 299–303.

Bardo, M.T., Neisewander, J.L., 1986. Single-trial conditioned place preference using intravenous morphine. Pharmacol. Biochem. Behav. 25, 1101–1105.

Bilsky, E.J., Marglin, S.H., Reid, L.D., 1990. Using antagonists to assess neurochemical coding of a drug's ability to establish a conditioned place preference. Pharmacol. Biochem. Behav. 37, 425–431.

D'Amour, F.E., Smith, D.L., 1941. A method for determining loss of pain sensation. J. Pharmacol. Exp. Ther. 72, 74–79.

Daniels, D.J., Lenard, N.R., Etienne, C.L., Law, P.Y., Roerig, S.C., Portoghese, P.S., 2005. Opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a bivalent ligand series. Proc. Natl. Acad. Sci. U. S. A. 102, 19208–19213.

Dewey, W.L., Harris, L.S., Howes, J.F., Nuite, J.A., 1970. The effect of various neurohumoral modulators on the activity of morphine and the narcotic

antagonists in the tail-flick and phenylquinone tests. J. Pharmacol. Exp. Ther. 175, 435–442.

Fenu, S., Spina, L., Rivas, E., Longoni, R., Di Chiara, G., 2006. Morphine-conditioned single-trial place preference: role of nucleus accumbens shell dopamine receptors in acquisition, but not expression. Psychopharmacology (Berl) 187, 143–153.

Frances, H., Le Foll, B., Diaz, J., Smirnova, M., Sokoloff, P., 2004. Role of DRD3 in morphine-induced conditioned place preference using drd3-knockout mice. NeuroReport 15, 2245–2249.

Fundytus, M.E., Schiller, P.W., Shapiro, M., Weltrowska, G., Coderre, T.J., 1995. Attenuation of morphine tolerance and dependence with the highly selective delta-opioid receptor antagonist TIPP[psi]. Eur. J. Pharmacol. 286, 105–108.

George, S.R., Fan, T., Xie, Z., Tse, R., Tam, V., Varghese, G., O'Dowd, B.F., 2000. Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. J. Biol. Chem. 275, 26128–26135.

Gomes, I., Jordan, B.A., Gupta, A., Trapaidze, N., Nagy, V., Devi, L.A., 2000. Heterodimerization of mu and delta opioid receptors: a role in opiate synergy. J. Neuroscience 20, RC110.

Grabus, S.D., Martin, B.R., Brown, S.E., Damaj, M.I., 2006. Nicotine place preference in the mouse: influences of prior handling, dose and strain and attenuation by nicotinic receptor antagonists. Psychopharmacology (Berl) 184, 456–463.

Hepburn, M.J., Little, P.J., Gingras, J., Kuhn, C.M., 1997. Differential effects of naltrindole on morphine-induced tolerance and physical dependence in rats. J. Pharmacol. Exp. Ther. 281, 1350–1356.

Ide, S., Minami, M., Satoh, M., Uhl, G.R., Sora, I., Ikeda, K., 2004. Buprenorphine antinociception is abolished, but naloxone-sensitive reward is retained, in mu-opioid receptor knockout mice. Neuropsychopharmacology 29, 1656–1663.

Kim, H.S., Park, W.K., Jang, C.G., Oh, K.W., Kong, J.Y., Oh, S., Rheu, H.M., Cho, D.H., Kang, S.Y., 1997. Blockade by naloxone of cocaine-induced hyperactivity, reverse tolerance and conditional place preference in mice. Behav. Brain Res. 85, 37–46.

Lenard, N.R., Roerig, S.C., 2005. Development of antinociceptive tolerance and physical dependence following morphine i.c.v. infusion in mice. Eur. J. Pharmacol. 527, 71–76.

Lenard, N.R., Moore, J.B., Daniels, D.J., Portoghese, P.S., Roerig, S.C., 2005. Mu-delta agonist–antagonist opioid bivalent ligands produce antinociception without inhibition of gastrointestinal transit (GIT). FASEB J. 19 (Part 1 Suppl. S), A98-A98.

Lu, L., Grimm, J.W., Dempsey, J., Shaham, Y., 2004. Cocaine seeking over extended withdrwal periods in rats: different time courses of responding induced by cocaine cues versus cocaine priming over the first 6 months. Psychopharmacology (Berl) 176, 101–108.

Manzanedo, C., Serrano, A., Aguilar, M.A., Rodriguez-Arias, M., Minarro, J., 2001. Effects of CGS 10746B on hyperactivity and place preference induced by morphine. Behav. Brain Res. 126, 23–32.

Matthes, H.W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., Befort, K., Dierich, A., Le Meur, M., Dolle, P., Tzavara, E., Hanoune, J., Roques, B.P., Kieffer, B.L., 1996. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. Nature 383, 819–823.

Menkens, K., Bilsky, E.J., Wild, K.D., Portoghese, P.S., Reid, L.D., Porreca, F., 1992. Cocaine place preference is blocked by the delta-opioid receptor antagonist, naltrindole. Eur. J. Pharmacol. 219, 345–346.

Mucha, R.F., Walker, M.J., 1987. Aversive property of opioid receptor blockade in drug-naive mice. Psychopharmacology (Berl) 93, 483–488.

Mueller, D., Perdikaris, D., Stewart, J., 2002. Persistence and drug-induced reinstatement of a morphine-induced conditioned place preference. Behav. Brain Res. 136, 389–397.

Olson, V.G., Heusner, C.L., Bland, R.J., During, M.J., Weinshenker, D., Palmiter, R.D., 2006. Role of noradrenergic signaling by the nucleus tractus solitarius in mediating opiate reward. Science 311, 1017–1020.

Pchelintsev, M.V., Gorbacheva, E.N., Zvartau, E.E., 1991. Simple methodology of assessment of analgesics' addictive potential in mice. Pharmacol. Biochem. Behav. 39, 873–876.

Piepponen, T.P., Kivastik, T., Katajamaki, J., Zharkovsky, A., Ahtee, L., 1997. Involvement of opioid mu 1 receptors in morphine-induced conditioned place preference in rats. Pharmacol. Biochem. Behav. 58, 275–279.

Ribeiro Do Couto, B., Aguilar, M.A., Manzanedo, C., Rodriguez-Arias, M., Minarro, J., 2003. Reinstatement of morphine-induced conditioned place preference in mice by priming injections. Neural Plast. 10, 279–290.

Semenova, S., Kuzmin, A., Zvartau, E., 1995. Strain differences in the analgesic and reinforcing action of morphine in mice. Pharmacol. Biochem. Behav. 50, 17–21.

Shaham, Y., Shalev, U., Lu, L., De Wit, H., Stewart, J., 2003. The reinstatement model of drug relapse: history, methodology and major findings. Psychopharmacology (Berl) 168, 3–20.

Shalev, U., Grimm, J.W., Shaham, Y., 2002. Neurobiology of relapse to heroin and cocaine seeking: a review. Pharmacol. Rev. 54, 1–42.

Shippenberg, T.S., Heidbreder, C., 1995. The delta-opioid receptor antagonist naltrindole prevents sensitization to the conditioned rewarding effects of cocaine. Eur. J. Pharmacol. 280, 55–61.

Shippenberg, T.S., Bals-Kubik, R., Herz, A., 1987. Motivational properties of opioids: evidence that an activation of delta-receptors mediates reinforcement processes. Brain Res. 436, 234–239.

Sora, I., Elmer, G., Funada, M., Pieper, J., Li, X.F., Hall, F.S., Uhl, G.R., 2001. Mu opiate receptor gene dose effects on different morphine actions: evidence for differential in vivo mu-opioid receptor reserve. Neuropsychopharmacology 25, 41–54.

Suzuki, T., Ikeda, H., Tsuji, M., Misawa, M., Narita, M., Tseng, L.F., 1997. Antisense oligodeoxynucleotide to delta opioid receptors attenuates morphine dependence in mice. Life Sci. 61, PL 165–PL 170.

Suzuki, T., Yoshiike, M., Mizoguchi, H., Kamei, J., Misawa, M., Nagase, H., 1994. Blockade of delta-opioid receptors prevents morphine-induced place preference in mice. Jpn. J. Pharmacol. 66, 131–137.

Tzschentke, T.M., 1998. Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. Prog. Neurobiol. 56, 613–672.

Wang, B., Luo, F., Zhang, W.T., Han, J.S., 2000. Stress or drug priming induces reinstatement of extinguished conditioned place preference. NeuroReport 11, 2781–2784.

Xu, N., Wang, L., Wu, C., Pei, G., 2001. Spatial learning and morphine-rewarded place preference negatively correlates in mice. Pharmacol. Biochem. Behav. 68, 389–394.

Zhu, Y., King, M.A., Schuller, A.G., Nitsche, J.F., Reidl, M., Elde, R.P., Unterwald, E., Pasternak, G.W., Pintar, J.E., 1999. Retention of supraspinal delta-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. Neuron 24, 243–252.