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Delta Opioid Antagonist, 16-Me Cyprenorphine, Selectively Attenuates Conditional Fear- and DPDPE-Induced Analgesia on the Formalin Test

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FANSELOW, M. S., D. J. CALCAGNETTI AND F. J. HELMSTETTER. *Delta opioid antagonist, 16-Me cyprenorphine, selectively attenuates conditional fear- and DPDPE-induced analgesia on the formalin test.* PHARMACOL BIOCHEM BEHAV 32(2) 469–473 1989.—The effects of 16-Me cyprenorphine (M80) on the antinociception produced by reexposing rats to a chamber associated with footshock (1 mA, 0.75 sec) 24 hr earlier was assessed with the formalin test. In Experiment 1, intracerebroventricular administration of M80 dose-dependently (0.5–8 μg) reversed conditional analgesia. Experiment 2 demonstrated that M80 (5 μg) had no effect on baseline pain sensitivity, but completely reversed conditional analgesia. Experiment 3 demonstrated that 0.25 μg DAGO, 3.5 μg DPDPE, and 28 μg U50,488H all produced equivalent levels of antinociception on the formalin test. The 5 μg dose of M80 completely reversed the antinociception produced by DPDPE but did not influence that produced by DAGO or U50,488H. These data suggest, that at the doses employed, M80 is a selective δ-opioid receptor antagonist and that δ-receptors are involved in conditional fear-induced analgesia.

Rat Conditioned fear Delta-receptor Mu-receptor Kappa-receptor DAGO DPDPE U50,488H Stress-induced analgesia Formalin test 16-Me cyprenorphine (RX 8008M) Endogenous opioids Pavlovian conditioning

STRESS-INDUCED analgesia refers to the dramatic reduction in pain sensitivity engendered by a variety of stressful events (2, 18, 28). However, even in the absence of primary stressors, environmental cues previously associated with stress can modulate reactivity to nociceptive stimulation. For example, exposure to Pavlovian conditional fear stimuli cause a reduction in pain sensitivity as assessed by a variety of assays of nociception (6, 12, 23, 25, 30). There is substantial evidence that endogenous opioids are involved in the mediation of this conditional analgesia. Conditional fear stimuli reduce binding of radiolabelled opiate agonists (7,10). Tolerance to the analgesic effects of conditional fear stimuli can be produced by prior experience with systemic morphine (29). Furthermore, conditional fear stimuli can potentiate morphine antinociception (26). Finally, systemic administration of the opioid antagonists naloxone (13, 16, 20, 29, 30), naltrexone (14,20) and MR2266 (15) have all been shown to reverse this learned change in pain sensitivity. This opioid involvement appears to be central because peripheral administration of naltrexone methobromide, which does not readily pass the blood-brain barrier, does not affect conditional analgesia (5). However, intracerebroventricular (ICV) administration of naltrexone methobromide (5) or intrathecal administration of naloxone hydrochloride (29) can attenuate this environmentally produced antinociception.

The opioid antagonists that have been shown to reverse conditional analgesia (naloxone, naltrexone, and MR2266) predominantly act at the μ-opioid receptor and have less affinity for the δ-opioid receptor in vitro (27). Therefore, the present experiments examined 16-Me cyprenorphine (N-cyclopropylmethyl-6, 14-endoetheno-7 (alpha 1-hydroxy-1-methyo-16 alpha-methyl-6,7,8,14-tetrahydro nororipavine hydrochloride; RX 8008M), a reversible competitive opioid antagonist with high affinity for, and some selectivity to, the δ-receptor in vitro (27). The first two experiments used this compound in tests of conditional fear-induced analgesia (14).

In addition to its activity at δ-receptors, RX 8008M (M80) appears to have some antagonistic action at μ-opioid recep-

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tors both in vitro (27) and on urinary output in vivo (3). Previous work suggests that M80 is without action at the κ-opioid receptor (3,27). To determine the relative specificity of M80 to δ-receptors using our analgesiometric procedures, Experiment 3 examined this drug's ability to reverse the antinociception produced by three highly selective opioid receptor agonists, [D-Ala^2,-NMe PHe^4, Gly-ol^5]-enkephalin (DAGO), [D-Pen^2, D-Pen^5] enkephalin (DPDPE), and trans-3,4 dichloro-N-methyl-N-(2-(1-pyrrolidinyl) cyclohexyl benzeneacetamide (U50,488H). These three agonists are highly selective for μ, δ and κ-receptors, respectively (17,21).

As our index of pain sensitivity, we used a modified form of the formalin test (11). In this test, rats are given an injection of a dilute formalin solution into a hind paw and then observed for stereotyped paw lifting and licking responses directed at the site of the formalin injection. This procedure is highly sensitive to both conditional fear-induced (14) and opioid agonist-induced analgesia (1, 4, 24).

METHOD

Subjects

The subjects in all three experiments were naive adult female rats of Long-Evans descent weighing between 220–320 g. The rats were maintained in a colony room (14:10 light:dark cycle with dark onset at 1900 hr) and individually housed in hanging stainless steel cages with ad lib access to food (Prolab 3000) and tap water. All experimental procedures were conducted during the light portion of the cycle. The rats were handled daily, starting 4 days prior to surgery.

Surgery

Aseptic stereotaxic surgery was conducted under anesthesia induced by ketamine hydrochloride (100 mg/ml/kg) supplemented with sodium pentobarbital (0.03 ml injection of 50 mg/ml). With the skull leveled between lambda and bregma, an outer cannula guide (22 gauge stainless steel, Plastic Products, Roanoke, VA) was implanted into the right lateral ventricle (coordinates: A-P, 0.5 mm, M-L 1.5 mm, and D-V 3.2 mm from the surface of the cortex). The subjects were given a minimum of seven days to recover following surgery. During this time the rats were given daily handling that consisted of removing and cleaning each cannula plug wire with 70% methanol and adapting them to transportation from the colony to the laboratory.

Apparatus and Drugs

Conditioning and observation took place in one of four chambers (23.5×29×19.5) each of which was housed in a sound attenuating chest. A 7.5 W white light bulb illuminated each chamber. A clear plastic window, 30×30 cm, located in the front wall of the sound attenuating chest, allowed the experimenter to observe the subject's behavior. Ventilation fans provided background noise at 73 dB (C scale). Ammonium hydroxide solution (5%) was used to clean the chambers after each rat.

M80, a gift from Dr. C. F. C. Smith (Reckitt and Colman), was dissolved in slightly acidic (pH=5.5) pyrogen filtered (Millex-GV 0.22 μm, Millapore Corp.) isotonic saline. DPDPE (Bachem Inc.), DAGO (Bachem Inc.) and U50,488H (U50; a gift from Dr. P. VonVoigtlander, Upjohn Co.) were dissolved in pyrogen filtered, distilled water. The pyrogen filtered distilled water or pH adjusted isotonic saline vehicle served as control injections.

Behavioral Procedures

There were three independent experiments. The first provided a dose response function for M80's effects on conditional analgesia. The second added a no shock control condition. The third examined M80's effect on the antinociception produced by the three opioid agonists. The experiments followed the same general procedure consisting of two phases, Conditioning and Testing, that took place 24 hr apart. All the experiments used independent groups designs with subjects randomly assigned to groups.

On the conditioning day of Experiment 1, all the rats were placed in an observation chamber and 4 min later they received 3 shocks (1 mA, 0.75 sec) spaced 20 sec apart. Twenty sec after the last shock they were returned to their home cage. Half the rats of Experiment 2 received this shock treatment with the only change being that the intershock interval was increased to 60 sec, the other half were exposed to the chamber for an equivalent amount of time but received no shock. For the third experiment all the rats were exposed to the chamber for 8 min on the conditioning day but they never received any shock.

The next day was the test day and the rats in all experiments received a 0.05 ml SC injection of 15% formalin under the dorsal surface of the right hind paw. This was followed 20 min later by an ICV injection of 4 μl of vehicle over 10 sec. For Experiment 1, this injection contained either 0, 0.5, 2, or 8 μg of M80. For the other experiments the injection contained either 0 or 5 μg M80, in a factorial combination with the other treatment conditions of the experiment (shock or no-shock for Experiment 2; agonist treatment for Experiment 3). In Experiment 3, the rats were given a second ICV injection 40 sec after the first. This injection contained either distilled water, 3.5 μg DPDPE, 0.25 μg DAGO, or 28 μg U50. This injection consisted of 10 μl delivered over 20 sec.

All ICV drug injections were conducted by backloading the appropriate solution through a 28 gauge internal cannula (Plastic Products, Roanoke, VA) into PE-50 tubing (Intramedic No. 7411). The internal cannula was cut to extend 0.5 mm beyond the guide cannula. Fluid was delivered by a 100 μl Hamilton syringe connected to a repeating dispenser. After completing the drug injection the inner cannula was allowed to remain in place for at least 15 sec. Rats were gently restrained by hand during the injection procedures.

Ten min following the ICV injection procedure, the rats were placed in the chamber for an 8 min observation period. During this time, every 8 sec each rat's behavior was scored with a time sampling procedure similar to that described previously (13). A metronome provided the observer with a pacing signal that indicated when an observation should be made. The behavior occurring at the instant of the observation was judged to be either formalin-induced paw lifting, formalin-induced paw licking or general activity. In paw lifting, the formalin-treated paw is raised and held elevated close to the rat's body. Paw licking is any licking or contact of the treated paw with the animal's mouth (13). All other behaviors, such as locomotion and grooming, were scored as general activity. The observer was blind to the treatment conditions.

Histology

Histological verification of cannula placement consisted of overdosing the subjects with sodium pentobarbital and injecting them ICV with 2 μl of ink. Following the ink (5–15 min) they were perfused transcardially with saline followed

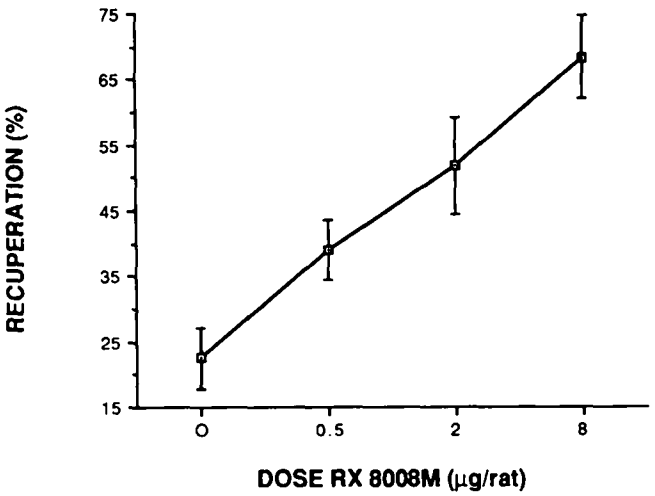

FIG. 1. The mean percentage of observations scored as formalin-induced recuperative behavior as a function of ICV dose of M80. The 0, 0.5, 2.0, 8.0 μg groups contained 6, 7, 7, 8 subjects, respectively. Unequal Ns were caused by the exclusion of subjects based on histology. All rats received 3 shocks (20 sec apart, 1 mA, 0.75 sec) 24 hr prior to the test.

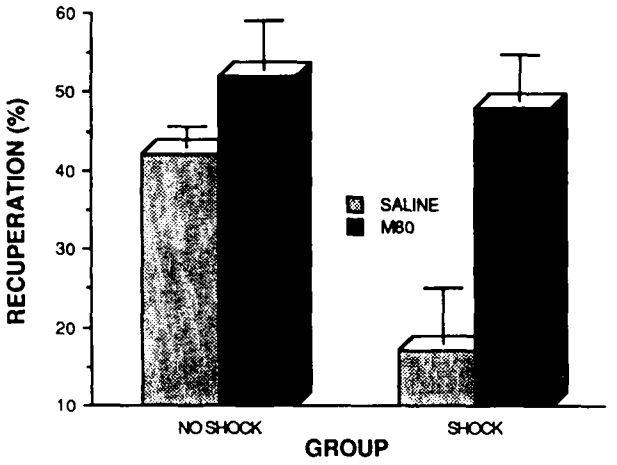

FIG. 2. The mean percentage of observations scored as formalin-induced recuperative behavior of rats given either 5 μg M80 or saline immediately prior to the formalin test. The rats received either shock or no shock 24 hr prior to the test. All groups contained 5 subjects.

by formalin (10%). The brains were removed and coronal sections were made along the cannula tract. Positive cannula placement was verified by the presence of ink in the ventricles. Only those subjects for which positive placement was verified were included in the analyses.

Data Analysis

Following Fanselow and Baackes (14), both types of formalin-related behavior were collapsed into a single category called recuperation for analysis. For each subject, a recuperative score was calculated as the percentage of samples scored as recuperation. As recuperative scores tend to have a Poisson distribution they were transformed to square roots for analysis (19). The data for each experiment was subjected to an overall Analysis of Variance, followed by a set of a priori planned comparisons.

RESULTS

Mean percentages of recuperative behavior as a function of M80 dose obtained in Experiment 1 are presented in Fig. 1. The ANOVA indicated reliable differences between groups, $F(3,23)=15.47$, $p<0.01$. Recuperative behavior increased linearly with dose, $F(1,23)=35.9$, $p<0.001$. This increase in recuperation suggests that M80 reversed conditional analgesia.

Since all the rats in Experiment 1 received fear conditioning, it is possible that M80 increased recuperative behavior by increasing baseline pain sensitivity, rather than reversing conditional analgesia. Additionally, the design of the first experiment did not allow us to determine how complete the reversal of conditional analgesia was. Therefore, Experiment 2, which included no shock controls, was conducted. Based on the data of the first Experiment, we selected a 5 μg dose of M80. Group means are presented in Fig. 2. A 2×2 ANOVA, conducted on the results of that experiment indicated that both the Shock, $F(1,16)=8.15$, $p<0.05$, and Drug, $F(1,16)=16.56$, $p<0.05$, main effects were reliable. Importantly, the interaction was also reliable, $F(1,16)=4.67$, $p<0.05$. Pairwise contrasts indicated that the rats that received both vehicle and shock recuperated less than the other three groups, F's$(1,16)>12.61$, p's<0.005, which did not differ, F's$(1,16)<1.2$, p's>0.28. These data indicate that rats exposed to shock 24 hr earlier displayed a conditional analgesia. As is made clear by Fig. 2, M80 completely reversed this analgesia but did not affect baseline pain sensitivity in animals that had not been shocked.

Rather than the antinociception produced by conditional fear stimuli, Experiment 3 examined the antinociception produced by ICV administration of three receptor selective opioid agonists. The doses of these agonists were based on prior work conducted with the formalin test (4) so that they would produce equal suppression of formalin-induced recuperative behavior. The M80 dose was that used in Experiment 2. Mean recuperative scores are presented in Fig. 3.

A 2 (M80 or H_2O) × 4 (H_2O, DPDPE, DAGO, or U50) factor ANOVA indicated a reliable main effect for agonist, $F(3,52)=15.53$, $p<0.001$. The main effect for antagonist was not reliable, $F(1,52)=2.92$, $p=0.09$. However, the Antagonist × Agonist interaction was statistically reliable, $F(3,7)=7.01$, $p<0.001$. As can be seen in Fig. 3, when the animals were not given the antagonist, all three agonists produced similar levels of analgesia. Statistically, planned comparisons indicated that the three agonist alone groups did not differ, F's$(1,52)\leq 1.27$, but combined, these three groups differed reliably from the H_2O+H_2O controls, $F(1,52)=22.39$, $p<0.001$. The only effect of M80 was that it completely reversed the antinociception produced by the δ-opioid agonist DPDPE; M80 increased recuperative behavior in DPDPE-treated rats, $F(1,52)=22.82$, $p<0.001$. As was found in Experiment 2, the antagonist had no effect on baseline pain sensitivity as indicated by comparing the $M80+H_2O$ group to the H_2O+H_2O group, $F(1,52)<1$. M80 had no effect on the rats treated with the μ agonist DAGO, $F(1,52)<1$, or the κ agonist U50, $F(1,52)<1$.

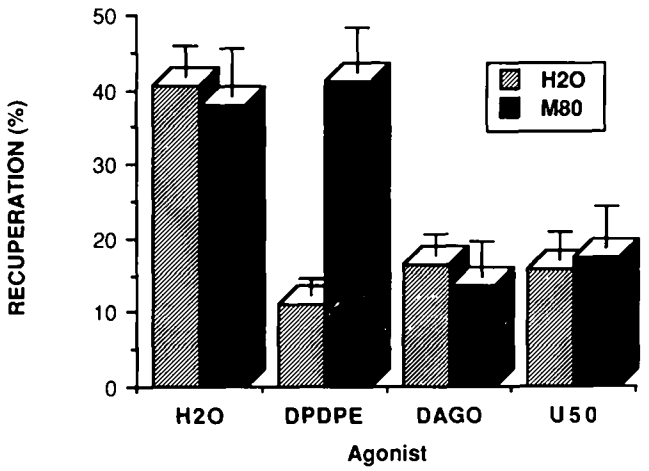

FIG. 3. Behavior scored as the mean percentage of recuperation in rats given ICV M80 (5 μg) or H_2O followed by a second ICV injection of H_2O, DPDPE (3.5 μg), DAGO (0.25 μg), or U50,488H (28 μg). All groups contained 7 subjects except the H_2O+H_2O, H_2O+U50, M80+H_2O, and M80+DAGO groups, which contained 10, 6, 8, 8, subjects, respectively.

DISCUSSION

In both Experiments 2 and 3 it was shown that M80 does not affect the level of formalin-induced recuperative behavior in animals that did not receive shock. Therefore, as with naloxone and naltrexone, M80 does not affect baseline pain sensitivity (13, 14, 24). However, Experiments 1 and 2 demonstrated that in the presence of cues that were associated with shock 24 hr earlier, M80 increases recuperative behavior. This indicates that M80 can reverse the conditional analgesia that is produced by shock associated cues. Indeed, Experiment 2 demonstrated that a 5 μg dose of M80 can completely reverse conditional analgesia, suggesting that this form of stress-induced analgesia is totally dependent on an endogenous opioid process. Again, this is similar to the results obtained with naloxone (13).

While M80 has a high affinity for the δ-receptor, there is some suggestion that it may also act as an antagonist at μ sites (3,27). In comparison to naltrexone, M80 shows much greater affinity for the δ-receptor but less affinity for the μ-receptor in vitro (27). Comparisons of the dose-effectiveness of M80 obtained here, with previously published work using quaternary naltrexone, suggest that M80 is more effective than quaternary naltrexone in reversing conditional analgesia and does so at a lower dose (5). Thus, the greater effectiveness of M80 suggests an involvement of δ-receptors in conditional analgesia. M80 shows virtually no affinity for κ-receptors either in vitro (27) or in vivo (3); therefore, it is unlikely that the effect of this drug on conditional analgesia is mediated at that receptor. Very strong corroborative support for this analysis is provided by Experiment 3. When we examined the antinociception produced by equipotent doses of highly selective opioid agonists, the 5 μg dose of M80 that completely reversed conditional analgesia, completely reversed the antinociception produced by the δ-agonist DPDPE but had no effect on DAGO or U50, agents that are selective for μ- and κ-receptors, respectively.

The results of Experiment 3 suggest that M80 can be used successfully as a selective δ-receptor antagonist. Previously, the peptide ICI-174864 (ICI) has served this role (9). However, M80 may prove to be a preferable compound as ICI has been found to cause neuronal injury that is not related to its opioid effects (22). Additionally, ICI may have weak partial δ agonist properties and it may degrade into a peptide that acts as a μ agonist (8).

A question of interest is whether M80's ability to reverse conditional analgesia derives from an action at the same population of receptors involved in the reversal of conditional analgesia that is produced by naloxone, naltrexone and MR2266. These other opioid antagonists all have some, albeit low, affinity for the δ-receptor. Therefore, it is possible that the action of all of these opioid antagonists upon conditional analgesia is mediated at a common population of δ-receptors. The greater potency of M80, relative to naloxone, naltrexone and MR2266, is consistent with this possibility. Alternatively, two pools of opioid receptors may be involved in conditional analgesia; one that is antagonized by naloxone or naltrexone and a second that is blocked by M80. This suggestion is supported by DeVries *et al.*'s (10) observation that conditional fear affects the binding of ^{3}H Leu-enkephalin to two classes of receptors, only one of which is antagonized by naloxone. With the procedures reported here, both M80 or naloxone can completely eliminate the conditional analgesia. Therefore, if two receptor populations are involved in this form of conditional analgesia, both populations appear to be necessary as there is no residual antinociception following either M80 treatment or naloxone treatment.

In conclusion, we find that the opioid antagonist 16-Me cyprenorphine (RX 8008M) reverses the antinociception produced both by conditional fear procedures and DPDPE but does not affect the antinociception produced by DAGO and U50,488H. This indicates that within the dose response range investigated here, this antagonist is selective for δ-receptors, and that δ-receptors are involved in conditional fear-induced analgesia.

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