Possible False Negative Results in Conditioned Place Preference Induced by Low-dose Morphine in Mice

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Abstract
Conditioned Place Preference (CPP) is an easy-to-run experiment that is widely used to detect the reinforcing property of drugs. Most CPP experiments have employed post-training tests without drug injection. Here, a post-training test with drug injection was employed. C57BL6 mice received CPP training with 0.3 mg/Kg or 3.0 mg/Kg morphine and were then tested three times: without morphine, with 0.3 mg/Kg morphine, and with 3.0 mg/Kg morphine. The mice trained with 0.3 mg/Kg did not show a significant increase in staying time at the drug-associated compartment in the drug-free test, but a significant increase was found in tests with the drug. The mice trained with 3.0 mg/Kg morphine showed conditioned preference in all tests and displayed enhancement of the conditioned preference in tests with the drug. The animals experienced environmental cues under the influence of the drug during the CPP training but were exposed to cues without the drug in the drug-free test. Thus negative results obtained in the post-training drug-free test may have been caused by differences in the stimuli the animals experienced.

Keywords: Conditioned place preference; Reinforcing property of drug; Drug addiction; Morphine

Introduction
One of the main issues in behavioral pharmacology is the reinforcing property of drugs. The reinforcing property is, by definition, detected by behavioral measurement. A straightforward method is self-administration of the drug through an implanted cannula. If the drug has a reinforcing property, a lever press or other operant behavior is maintained by contingent immediate administration of the drug. This method is applied mostly to monkeys and rats. An alternative measurement technique is Conditioned Place Preference (CPP), in which drug injection is associated with a particular environment, for example, a black compartment of an experimental chamber. The procedure is technically rather simple in comparison to self-administration, but rather complicated theoretically [1]. The CPP procedure is respondent conditioning in which the environment (e.g., a black compartment) is the CS (conditioned stimulus) and the drug injection is the UCS (unconditioned stimulus). In most CPP experiments, another environment (CS-) (e.g., a white compartment) is associated with vehicle injection; in this case, the CPP is differential conditioning using CS+ (CS associated with UCS) and CS- (CS not associated with UCS). The reinforcing property of the drug is expressed as the increase in staying time in the environment associated with the drug injection before and after the conditioning. That is, the conditioned response (CR) is staying time. One particular feature of CPP as respondent conditioning is that there is no measurement of unconditioned response (UR) before conditioning. In other words, the presence of the UR (the reinforcing property) is estimated by formation of the CR. Thus, in comparison to self-administration, demonstration of the reinforcing property is indirect. Staying time can also be considered as an operant behavior maintained by a conditioned reinforcer. In this explanation, CPP is a procedure that produces a conditioned reinforcer by respondent conditioning with the drug, and the reinforcing property is detected by the operand (staying).

In addition to the problem of biased and unbiased design of conditioning [2], the test condition after the conditioning must also be considered in CPP [1,3]. In the most common CPP procedure, the post-training test is carried out without the drug. That is, the testing procedure is an extinction procedure, presentation of the CS without the UCS. The CS is, however, presented under the drug-injected state during the conditioning; therefore the CS during the extinction test may be ‘subjectively’ different from the CS during the conditioning. Thus post-training tests with and without the drug may give different results.

Nomikos and Spyraki [4] have carried out post training tests with and without the drug after CPP with cocaine in rats; they found no difference between the two tests. Similarly, Spyraki et al. [5] did not find a difference between post-training tests with and without diazepam; however, they found conditioned place aversion by picrotoxin only in the post-training test with the drug injection. Morphine (1.0 mg/Kg)-induced CPP in rats was also not affected by drug injection prior to testing [6]; in some cases a prior injection even reduced the conditioned preference induced by 1.0 mg/Kg morphine [7]. Thus, there have been conflicting effects of pre-treatment by different drugs on post-conditioning tests.

There could, however, be a species difference between rats and mice. After CPP training in mice with 5, 10, and 20 mg/Kg morphine, Bespalov et al. [8] administered tests with different doses. They did not find conditioned preference in a drug-free test, but the conditioned preference emerged in tests with pre-treatment morphine. Other drugs -- heroin (0.1–3 mg/kg), fentanyl (0.01–0.3 mg/kg), cocaine (10–30 mg/kg) and pentobarbital (10–30 mg/kg) -- did not have such effects. Thus, the pre-treatment effect appears to be specific to the drug used for conditioning. Furthermore, the strongest conditioned preference was obtained at the dose used for the conditioning. The lack of CPP with these doses (5–20 mg/Kg) of morphine in the drug-free test is a rather
exceptional finding. One procedural peculiarity of Bespalov et al. [8] was their training procedure: They trained animals twice a day. They injected vehicle and placed the mice in one compartment and then injected morphine and placed the mice in another compartment. Thus, the subjects received morphine injections every day. In addition to this procedural difference, Bespalov et al. [8] used Swiss-Webster mice, which are rarely used for CPP experiments; strain differences in CPP with morphine have been previously reported [9]. In fact, Semenova et al. [9] showed successful CPP in C57/BL6 by a procedure similar to that of Bespalov et al. [8]. The important finding of Bespalov et al. [8] was conditioned preference in the test with drug pre-treatment but no conditioned preference in the drug-free test. Thus, the drug-free test may provide a false negative conclusion about CPP. Subsequently, Sakoori and Murphy [10] used 4 mg/Kg morphine for C57/BL6 mice and found CPP in the drug-free test as well as enhancement of conditioned preference in tests with morphine pre-treatment. But they reported no such enhancing effects with cocaine-induced CPP.

Tuazon [11] observed successful CPP after 1.0 mg and 3.0 mg/Kg morphine injections in mice but not after injection of 0.1 mg/Kg morphine. Kennedy et al. [12] failed to obtain CPP after 0.25 mg/Kg morphine injection. Examination of the effects of drug injection in the post-training test is therefore particularly important after CPP with a lower dose. If the drug influences CS during CPP training, subjects may display conditioned preference in the test with the drug injection but not in the test without the drug. Here, the reinforcing property of low dose morphine was examined using the CPP procedure. The post-training test was carried out with and without the drug. Two dosages were employed: 3.0 mg/Kg, which should cause CPP, and 0.3 mg/Kg, which should not cause clear conditioned preference in a conventional post-training test without the drug.

Materials and Methods

Animals and housing

Twenty-eight male C57/BL6J mice were obtained from the Nihon Bio-Supply Center (Tokyo, Japan) for this study. The mice were 8 weeks old at the start of the experiment. The mice lived in a room under reversed 12D/12L lighting conditions. Temperature was maintained at 24°C. Food and water were freely available. Mice were treated in accordance with guidelines established by the Japanese Society for Animal Psychology.

Apparatus

A conventional CPP apparatus (ENV3015, MED) was used. The apparatus had three compartments. One compartment had a grid floor and black walls (16×13×12 cm), the second had a stainless steel mesh floor and white walls (16×13×12 cm), and the third had a flat grey floor and grey walls (6×13×12 cm). The grey compartment was the center compartment, and it was connected with the other two via guillotine doors. Each compartment had a ceiling lamp. Luminance below the lamp was 11.4, 11.0, and 10.9 lx in the white, grey, and black compartments, respectively.

Behavioral procedures

Mice were divided into two groups: 3.0 mg/Kg group and 0.3 mg/Kg group. Each group consisted of 14 mice. The 3.0 mg group received injection of 3.0 mg/Kg morphine and the 0.3 mg group injection of 0.3 mg/Kg during CPP training.

Days 1 and 2 (pre-training test): Each mouse was placed in the center compartment. After 5 min, the doors to the other compartments were opened and the animal was allowed to move freely throughout the apparatus for 15 min. The amount of time spent in each compartment during the day 2 trial was used as the baseline. The floor and walls of each compartment were wiped with 70% ethanol after each trial.

Days 3–8 (CPP training): The mice received injections of morphine on days 3, 5, and 7, and saline on days 4, 6, and 8. On drug-treatment days, subjects were injected with morphine and restricted to either the black or white compartment for 40 min. On saline-treatment days, these subjects were injected with saline and restricted to the other compartment for 40 min. Compartment choice following drug administration was selected in an unbiased way (i.e., the baseline compartment preference did not affect this selection process).

After the conditioning, the subjects received the three tests in random order; between the tests, they received two days of conditioning: one day of the drug treatment and one day of the saline treatment.

No-drug test: This was exactly the same as the day 1/day 2 test (i.e., 5 min in the grey compartment, followed by 15 min of unrestricted movement). The amount of time spent in each compartment was measured during the period of unrestricted movement.

3.0 mg/Kg test: The mice were injected with 3.0 mg/Kg morphine and received a test identical to the no-drug test.

0.3 mg/Kg test: The mice were injected with 0.3 mg/Kg morphine; the test was identical to the 3.0 mg/Kg test.

Pharmacological procedures

Morphine HCl (Dainippon Sumitomo Pharma, Osaka, Japan Seiyaku) was dissolved in physiological saline. Doses were 0.3 or 3.0 mg/Kg in 10 ml/Kg of saline. Intra-peritoneal injections were used to administer the drug or saline. Following the injection, animals were immediately placed into the CPP apparatus.

Statistical analysis

The amount of time spent in the drug-associated compartment was measured to detect effects of CPP training. Planned multiple comparisons were conducted [13] and ANOVA was therefore not carried out. Because the purpose of the experiment was to evaluate the presence of CPP, the baseline was compared with the three tests using Holm’s correction for multiple comparisons.

Results

Figure 1a shows the amount of time spent in the drug-associated compartment before and after conditioning in the 0.3 mg/Kg group. To confirm CPP, the amount of time spent in the drug-associated compartment after the conditioning was compared with that before the conditioning (two-tailed paired t-test with Holm’s correction). The staying time after the CPP training test without drug injection did not significantly differ from the staying time of the baseline (paired t-test, t(14)=1.71, P<0.10). Thus, the reinforcing property of the 0.3 mg/Kg morphine injection was not obtained with this test. The post-training test with the 0.3 mg/Kg morphine injection, however, differed from the baseline (paired t-test, t(14)=2.75, P<0.05). Thus, the reinforcing property was confirmed. The post-training test with the 3.0 mg/Kg morphine injection also resulted in an increase in staying time at the drug-associated compartment, but the difference was not statistically significant after Holm’s correction (paired t-test, t(14)=2.48, P=0.54). There were considerable individual differences in the test with 3.0 mg/Kg injection. Five mice showed a peak of conditioned preference at 0.3 mg/kg injection, while the other 9 mice showed a peak at 3.0 mg/kg injection.
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Thus, the mice showed conditioned preference (t(14)=6.08, P<0.001). Thus, the mice showed conditioned preference (t(14)=3.16, P<0.01) and between the baseline and the 3.0 mg/Kg test (t(14)=3.06, P<0.01), between the baseline and the 0.3 mg/Kg test (t(14)=3.14, P<0.001). The subgroup with the peak at 0.3 mg/Kg also showed a significant increment at the peak (t(11)=2.96, P<0.05) from 0.3 mg/Kg but the subgroup with a peak at 0.3 mg/Kg did not show a significant increment there (t(3)=0.91, P=0.46) from 3.0 mg/Kg.

The most important finding in the present experiment is the dependency of the conditioned preference of low-dose morphine on the test procedure. Conditioned preference was demonstrated in the post-training test with drug injection even at a training dose that resulted in no conditioned preference in the drug-free test.

C57/BL6 mice did not demonstrate conditioned place preference in a post-training test without morphine after CPP training with 0.1 mg/Kg [11], 0.25 mg/Kg [12], or even 0.5 mg/Kg [14]. Thus the present results of the post-training drug-free test after CPP training with 0.3 mg/Kg agree with previous reports. Because the preference demonstrated by the test after the drug injection indicates the reinforcing property of 0.3 mg/Kg morphine, the drug-free test may have produced false negative results. As noted in the introduction, the environmental cues (CS+) were cues under the drug-injected state, and cues without drug influence might have differed from the original CS+ for the animals. The CS+ functioned as a conditioned reinforcer during the post-training-tests, and its reinforcing value became weaker without the influence of the drug. An interesting finding is that some mice showed stronger conditioned preference after 0.3 mg/Kg than after 3.0 mg/Kg. This phenomenon agrees with the observations of Bespalov et al. [8], who reported that mice displayed the conditioned preference most strongly in the test with the training dose of morphine. Dose response curves of morphine at the post-training test had peaks at the training doses [8]. This phenomenon is similar to stimulus generalization after conventional discriminative training, supporting the explanation based on stimulus control presented in the introduction. Unfortunately, there has been no report of 0.3 mg/Kg morphine discrimination in mice; however, ED50 of dose generalization after discrimination of 3.0 mg/Kg in mice was 1.12-1.32 mg/Kg [15,16]. Rats trained on discrimination of 3.0 mg/Kg morphine showed ED50 of 0.17 mg/Kg [17] or 0.89 mg/Kg [18]. Thus, it is plausible to assume that there is a subjective difference between 0.3 mg/Kg and 3.0 mg/Kg morphine in mice.

The results of CPP with 3.0 mg/Kg morphine also support the idea that the post-training test with drug injection is a sensitive procedure to detect reinforcing property of the drug. This enhancing effect agrees with results of Sakoori and Murphy [10], in which a higher dose (4 mg/Kg) was used. Unpredicted was the peak preference at 0.3 mg/Kg displayed by 3 mice, although their preference did not differ after 3.0 mg/Kg and 0.3 mg/Kg injection. Thus, the preference at 0.3 mg/Kg was probably attributable to variability of the data.

In conclusion, the present experiment suggests the possibility of false negative results as a consequence of post-training test without drugs. To generalize this conclusion, more experiments with different types of drugs are required.

Discussion

mg/Kg injection. Figures 1b and c show separate results for these two subgroups of mice. The subgroup with the peak at 3.0 mg/Kg showed a significant difference in staying time between the test after 0.3 mg/Kg injection and that after 3.0 mg/Kg injection (paired t-test, t(9)=5.48, P<0.001). The subgroup with the peak at 0.3 mg/Kg also showed a significant difference (paired t-test, t(4)=3.14, P<0.05).

Figure 2a shows results for the 3.0 mg/Kg group. There was a significant difference between the baseline and the test without the drug (t(14)=3.06, P<0.01), between the baseline and the 0.3 mg/Kg test (t(14)=3.16, P<0.01) and between the baseline and the 3.0 mg/Kg test (t(14)=6.08, P<0.001). Thus, the mice showed conditioned preference for the drug-associated compartment after conditioning with 3.0 mg/Kg morphine injection in all tests. Three subjects in this group had a peak at 0.3 mg/Kg, and the remaining 11 subjects had a peak at 3.0 mg/Kg. Figures 2b and 2c show averages of these two subgroups. The subgroups with a peak at 3.0 mg/Kg showed a significant increment at the peak (t(11)=2.96, P<0.05) from 0.3 mg/Kg but the subgroup with a peak at 0.3 mg/Kg did not show a significant increment there (t(3)=0.91, P=0.46) from 3.0 mg/Kg.

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Acknowledgement

This research was supported by Aid for Scientific Research on Innovative Area (25118001).
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