

Original Article

Morphine-induced conditioned place preference and associated behavioural plasticity in HIV-1 transgenic rats

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Abstract: The prevalence of morphine addiction in HIV-1 infected persons is higher than the healthy population. The mu-opioid receptor (MOR) which mediates the actions of morphine is shown to be up-regulated in the HIV-1 transgenic (HIV-1Tg) rat. In this study, we used the conditioned place preference (CPP) test to investigate if HIV-1Tg rats are more sensitive to the addictive properties of morphine compared to F344 control animals. Morphine-CPP was successfully established in the HIV-1Tg and F344 rats at the dose of 3.5 mg/kg. Interestingly, the animals that had morphine paired with one side of the two-chamber CPP apparatus (the white chamber) failed to show any decline in preference for the morphine-paired chamber after 17 to 20 days of extinction testing. An analysis of the change in preference that occurs within a session as a result of the habituation of exploratory behaviour suggested that morphine enhanced a natural preference for the white chamber. We suggest that the morphine CPP procedure may sometimes result in a behavioural plasticity that does not conform to the predictive learning model of classical conditioning and may reflect a form of associative learning known as evaluative conditioning. The animals that had morphine paired with the other chamber (the black chamber) showed extinction. Moreover greater resistance to extinction was observed in the HIV-1Tg rats. Following extinction the HIV-1Tg and F344 groups were each divided into two groups. One group was tested for reinstatement following a 1 mg/kg and 3.5 mg/kg morphine prime on 2 consecutive days, respectively. The second group was tested for reinstatement after exposure to foot shock. Reinstatement with drug-prime or with the foot shock stressor was not observed.

Keywords: Morphine, HIV-1Tg rat, conditioned place preference (CPP) test, mu-opioid receptor, behavioural plasticity

Introduction

Morphine is used as a pain-killer in the management of diseases like cancer and acquired immunodeficiency syndrome (AIDS). Morphine can produce a feeling of euphoria and a state of well-being; this makes it a popular choice to be used as a recreation drug which is often abused [1]. Approximately one third of the AIDS cases in the USA have been suggested to be related to the use of injected addictive drugs, such as morphine, making it a very significant co-factor in contracting human immunodeficiency virus type 1 (HIV-1) [2, 3]. The reason for higher addiction rates to morphine among HIV-1 infected individuals may be to overcome the depression and stress due to the disease or that morphine addicted persons may be more prone to risky sexual behaviour and in turn be more prone to

contracting HIV-1 infection [4, 5]. Conversely, HIV-1 infected persons are more prone to be addicted to morphine than the general population [6]. Thus, there could exist a bidirectional and synergistic relationship between morphine abuse and the contraction of HIV-1 infection.

HIV-1-induced changes in the sensitivity to the addictive properties of drugs are difficult to establish in human studies which are primarily correlational. Animal models allow for experimental tests of this hypothesis. The HIV-1 transgenic (HIV-1Tg) rat is a useful animal model to study HIV-1 infection as it expresses the transgene consisting of an HIV-1 provirus with functional deletion of *gag* and *pol* genes [7]. The rat expresses seven of the nine HIV-1 viral proteins [7]. Compared to F344 control animals the HIV-1Tg rat has a lower body-weight throughout life

but has no signs of anhedonia. Only at older ages they begin to show clinical signs of wasting that progress to death at an earlier age [8-11]. The HIV-1Tg rat displays the characteristics of HIV associated dementia (HAD); it shows impaired spatial learning and memory, reversal learning and the learning of a new strategy compared to control F344 rats [12, 13]. The persistent presence of HIV viral proteins may contribute to the dysregulation of both the dopaminergic and opiodergic pathways, leading to alterations in reward and addictive behaviours [14]. Expression of the dopamine receptor (D1R) is increased in HIV-1Tg rats and methamphetamine-induced behavior sensitization is more profound in the HIV-1Tg rats [15]. However, the HIV-1Tg rat shows less behavior sensitization to nicotine in comparison to F344 [16]. Both these studies suggest that the presence of HIV proteins in the HIV-1Tg rat altered its sensitivity to an addictive drug. The mu opioid receptor (MOR) which is expressed in the central nervous system cells and in the immune system cells, may contribute to the mediation of the analgesic and rewarding actions of opioids such as morphine [9, 17]. Exposure of peritoneal macrophages of F344 rats to serum from HIV-1Tg induces upregulation of MOR-expression, which is abolished by addition of gp120 antibody to the serum [9]. HIV-1Tg rats when treated with morphine show significantly longer tail flick latencies compared to control F344 rats, indicating that there is an increased potency of morphine's anti-nociceptive properties in the HIV-1Tg rats [18]. As MOR is up-regulated in the HIV-1Tg rat and it has also been shown that the anti-nociceptive properties of morphine are enhanced in the HIV-1Tg rat we wanted to investigate if the rewarding and addictive properties of morphine are also enhanced in the HIV-1Tg rat.

Craving for and relapse to a drug after a period of abstinence are the salient features of addiction [19]. In drug addiction there is a very significant behavioural change in that the addict has decreased ability to control drug seeking behaviour [20]. In laboratory animals relapse following abstinence is modelled using the reinstatement procedure. Animals are first trained to associate a behaviour (self administration) or a place (CPP) with a drug. Then a period of extinction is introduced whereby the behaviour or place is no longer followed by the drug and responding returns to baseline. The effects of various stimuli are then introduced to look for evidence of the

return of responding or reinstatement. Reinstatement of drug seeking behaviour, or relapse, is caused in the laboratory by re-introduction to the drug (drug-primed), exposure to stress (stress-primed) or re-introduction of the stimuli which was previously associated with drug use (cue-primed) [21, 22]. Re-introduction to previous stimuli/environment associated with drug-use increases the urge to use drugs [20]. These motivational properties of the conditioned stimuli associated with drug use can persist over a very long period of time, even after years of abstinence the conditioned stimuli can evoke drug-reinstatement [20].

The conditioned place preference (CPP) is a test commonly used in animals to measure the rewarding/addictive properties of a drug by measuring the motivational salience of drug-paired cues, by means of classical conditioning [23, 24]. In the CPP procedure, there is repeated pairing of a drug unconditioned stimulus with a distinct environment that serves as the conditioned stimulus and of a control vehicle in another environment [23]. The drug-paired environment gains motivational significance and the animal shows approach behaviour to that environment and a preference for it over the vehicle-paired environment in the drug-free state [23]. After the establishment of a CPP the animal is given free access to both environments for several sessions in the absence of the drug until the animal ceases to display a preference to the drug-paired environment (extinction) [23]. Extinction is evidence that the preference is a learned association [25]. However, extinguished associations are not permanently lost and can be re-expressed. Exposure to a drug-prime or to a stressor can be studied for their ability to reinstate the extinguished CPP response after extinction [23]. Reinstatement procedures cause the previous associations to be re-expressed. It has been clearly demonstrated that stress causes a greater propensity towards drug-seeking behaviour, which causes a greater chance to become addicted [26, 27] and puts the subject at a greater risk for relapse [28]. This phenomenon has been noted in animals as well. In rats, an increase in locomotor activity (behaviour response to drug) was noted in morphine-treated rats exposed to mild repeated foot-shock [29]. Exposure to foot-shock also reinstates morphine-induced CPP in rats [30].

Morphine-induced CPP has been studied using

F344 rats [31-33]. There exists a strain-based sensitivity to morphine-induced CPP; F344 rats are more sensitive to morphine and acquire morphine-induced CPP at lower doses and with fewer conditioning cycles than Lewis rats [32]. In this study we have hypothesized that the HIV-1Tg animal would be more sensitive to the morphine associated cues than the F344 rat due to the up-regulation of MOR in the presence of HIV-1 viral proteins. As the unbiased CPP procedure used in the present study is not favourable for establishing differences in rates of acquisition due to potential "ceiling" effects [34], we investigated the difference between HIV-1Tg and control, F344 rats, in terms of extinction and the vulnerability to re-expression of motivational salience of morphine paired cues by reinstatement procedures. A greater motivational salience of morphine paired cues would be expected to result in greater resistance to extinction [25] and/or greater reinstatement of a preference for the drug-paired environment. In addition we investigated the effectiveness of a non-specific stressor (uncontrollable foot-shock) in establishing a reinstatement effect.

Materials and methods

Animals

Male HIV-1 transgenic (HIV-1Tg) rats and Fisher/NHsd 344 (F344) control rats at age of 3-4 weeks were purchased from Harlan Laboratories (Indianapolis, IN). Animals were group housed in ventilated cages with a 12/12 hour day/night cycles (lights on at 7 AM) in a temperature controlled environment (21-22°C) with food and tap water available *ad libitum*. The animals were tested in the light phase of the day/night cycle (9AM-2PM). Animals were acclimatized for approximately 2 months and handled for 3 consecutive days for 1-2 minutes each before beginning testing. The animals were approximately 3 months old when the experiment was conducted on them. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University, South Orange, NJ.

Drug

Morphine sulfate was obtained from (Sigma, St. Louis, MO). Morphine was administered intraperitoneally (i.p.) and each morphine treatment was administered as a 3.5 mg/kg dosage dur-

ing conditioning phase and as a 1.0 or 3.5 mg/kg dosage during reinstatement phase.

CPP apparatus

A commercially available CPP apparatus (MED Associates, East Fairfield, VT) was used. The apparatus consisted of an 18cm x 20cm white chamber with a grid floor and an 18cm x 20cm black chamber with a rail floor connected by a side passage; this design ensures that the animal is always visible to the camera. The side passage in the middle of the two chambers had two doors on each chamber wall; the doors could be closed to isolate each chamber. The animal movement and time spent in each chamber was recorded by an infrared camera (Stoelting ANY-maze, Wood Dale, IL). ANY-maze software (Stoelting ANY-maze, Wood Dale, IL) was used to analyse the camera recordings to measure time spent in the each chambers.

Foot shock administration

A custom foot-shock administering apparatus 40 cm w x 40 cm d x 35 cm h, connected to an animal shocker, was purchased from Stoelting ANY-maze (Wood dale, IL). The apparatus was set to deliver foot shock to an animal in it intermittently, for 15 minutes, with an amplitude of 0.5 mA, and a 0.5 s duration, (off period 10-70 s, mean 40 s).

CPP, extinction and re-instatement procedure

There were five phases for this experiment. During the preconditioning phase (1 day), the animals were placed in the side passage with the door of the chambers open and allowed to explore the entire box for 15 minutes. In the conditioning phase (6 days), an approximately equal number of animals of each strain (HIV-1Tg and F344) were assigned to each type of chamber (white or black) as the morphine paired side. The animals were injected with 3.5 mg/kg morphine (dose based on pilot data) every other day and placed in the pre-assigned morphine-paired side and on the next day were injected with equivalent volume of saline and placed in the saline-paired side, with the chamber door closed for 30 minutes. In the post-conditioning phase (1 day) the rats received no drug or saline and were placed in the side passage and allowed free access to the entire box for 15 minutes. For the first 8 days of the extinction proce-

dures, the animals were put in the side passage and given free access to the box for 30 minutes. After the first 8 days of extinction (Extinction 1) testing there was no evidence of extinction so the extinction procedure was altered in the hope of accelerating extinction. Evidence suggests that handling and transportation cues contribute to contextual conditioning [35], thus during extinction day 9 through day 17 (Extinction 2) the extinction procedure was repeated twice daily for 15 minutes per session with an average interval of 2 hours and 17 minutes between the 2 sessions, to increase the number of exposures to the transportation cues without changing the duration of exposure to the context (30 minutes total). Dividing the extinction procedure into two separate sessions also permitted for the opportunity of spontaneous recovery (see Discussion). Moreover, the HIV-1Tg rats received 3 additional days of extinction (see Results). Following extinction the rats in each strain were distributed equally to either be assigned in the drug-induced reinstatement group or in the stress-induced reinstatement group. The rats in the drug-induced reinstatement group received 1 mg/kg morphine on reinstatement day 1 and were given free access to the entire box for 15 minutes; on re-instatement day 2 the rats were given 3.5 mg/kg morphine and were given free access to the entire box for 15 minutes. The rats in the stress-induced re-instatement group on day 2 of the re-instatement phase were administered foot-shock for 15 minutes and subsequently put in the CPP apparatus and given free access to the entire box for 15 minutes.

The movement of the rats in the CPP apparatus was tracked by the ANY-maze camera and software in all phases. The experiment was carried out daily.

Statistical analysis

The means and standard deviations of the absolute time spent by animals (for each strain) in each chamber were calculated in all phases of the experiment. Inspection of the extinction data indicated that the chamber that was paired with morphine had a substantial effect on preference behaviour. To evaluate the effect of color/flooring of the chambers on the acquisition and extinction of CPP the data was analysed with color of the morphine-paired chamber as a factor. Careful inspection of the data also

revealed systematic changes in preference within a test session suggesting that there was habituation of exploratory behaviour over time. To test this hypothesis the extinction sessions were separated into segments of five-minutes. Analysis of variance (ANOVA) was used to study the interaction of strain (F344, HIV-1Tg), phase (pre-conditioning, post-conditioning, extinction 1 & 2, and/or reinstatement), morphine-paired chamber (Black, White), drug (morphine-paired, saline-paired). A value of $p \leq 0.05$ was considered to be statistically significant.

All analyses were done using the statistical software SPSS Statistics 17.0 IBM).

Results

Morphine-induced place preference

Fourteen HIV-1Tg and 14 F344 rats were placed in the CPP apparatus and given free access for 15 minutes (pre-conditioning) to determine if either strain showed a preference for one of the chambers. As shown in **Figure 1** neither the HIV-1Tg rats nor the F344 rats showed a preference for either the black or white chambers in the pre-conditioning phase. After 6 sessions of conditioning with 3.5 mg/kg morphine, both HIV-1Tg and F344 rats showed a CPP (**Figure 1**). A Strain (F344, HIV-1Tg) x Phase (Pre, Post) x Drug (Morphine-Paired, Saline-Paired) mixed ANOVA yielded a significant Phase x Side interaction $F(1,26) = 31.03$, $p = .000$, such that more time was spent in the morphine-paired side than in the saline-paired side post-conditioning but not pre-conditioning. To counterbalance for the morphine/side pairings, the strains were divided so that approximately half received morphine paired with the black side and the remainder received morphine paired with the white side. In addition to the color difference, the two sides also had different floors (grid and rail). Since the rats were tested under dim red-light illumination the floor was a much more salient cue than the color, eliminating a natural bias for the black chambers [34]. Nevertheless, for convenience the sides are referred to as black or white. The absence of a side preference during the 15 min pre-conditioning session suggested that the CPP apparatus used in this study was an unbiased apparatus. However after introducing the extinction procedure it was observed that there was a very high resistance to extinction in many animals. Inspection of the data indicated a

Morphine-induced CPP in HIV-1Tg rats

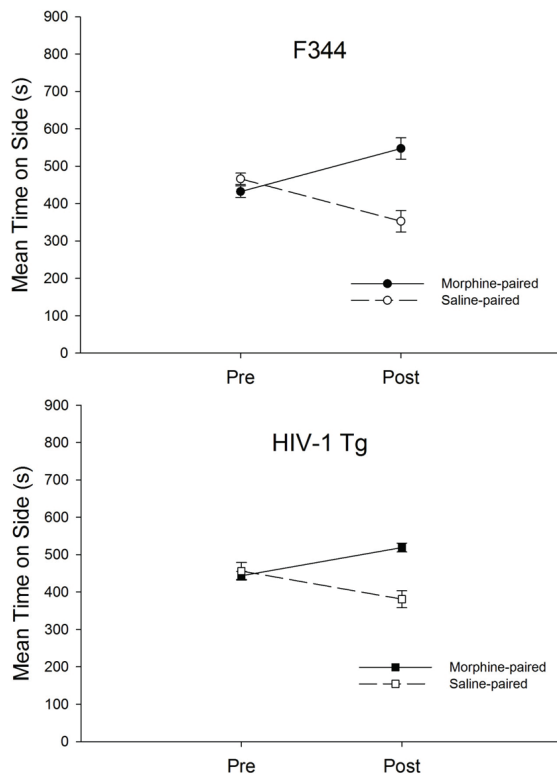


Figure 1. Time spent in the morphine-paired and saline-paired side of the CPP apparatus by strain in pre-conditioning and post-conditioning phase is shown. On post-conditioning day animals were conditioned daily for 15 minutes following a 3.5 mg/kg morphine (i.p.) dose every other day for 6 days. The following day after 6 conditioning sessions, animals were given free access to entire apparatus and tested for CPP.

stronger preference during extinction in the white-morphine paired animals than the black-morphine paired animals. Thus, to confirm that a CPP occurred in the white-paired and black-paired animals the data was analyzed with the counterbalancing factor (color of morphine-paired chamber) included as an independent variable (**Figure 2**). A Strain (F344, HIV-1Tg) x Phase (Pre, Post) x Morphine Color (Black, White) x Drug (Morphine-Paired, Saline-Paired) mixed ANOVA yielded a significant four way interaction, $F(1,24) = 4.59$, $p = .043$, that was due to a greater CPP when morphine was paired with the white side in the F344 animals [Phase x Drug x Morphine Color: $F(1,12) = 7.54$, $p = .018$] but not the HIV-1Tg rats. In the HIV-1Tg rats the significant CPP (Phase x Drug: $F(1,12) = 20.83$, $p = .001$) was not affected by the color of the morphine-paired chamber [Phase x Drug

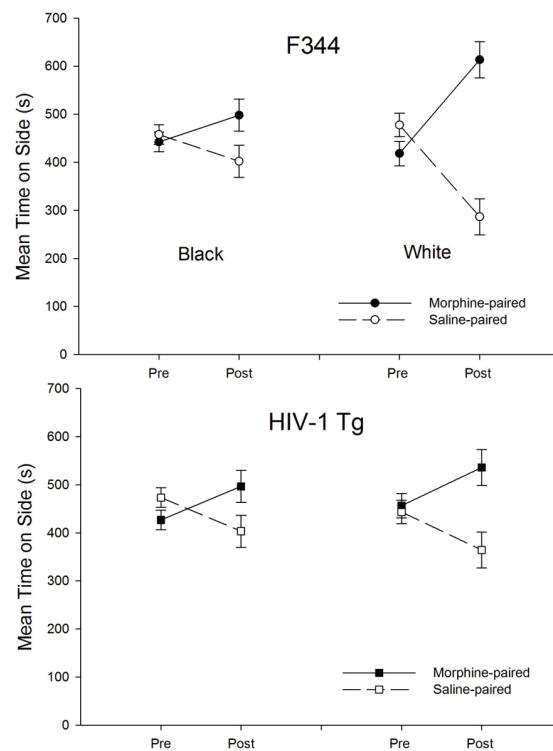


Figure 2. Time spent in the morphine-paired and saline-paired side of the CPP apparatus by strain and by color of morphine-paired chamber, in pre-conditioning and post-conditioning phase is shown.

x Morphine Color: $F(1,12) < 1$, $p = .784$] (**Figure 2**).

To investigate further the effect of time on the development of CPP we analysed the data in segments of 5-minutes. **Figure 3** shows the pre-conditioning and post-conditioning results separated into three 5-minute time segments. The rats were again divided in terms of the side that was paired with the drug (color of morphine-paired chamber). When the pre-conditioning data were analysed, no statistically significant effects were observed. However, the post-conditioning data shows clear changes in preference over the three, 5-minute periods. A Strain (F344, HIV-1Tg) x Phase (Pre, Post) x Morphine Color (Black, White) x Drug (Morphine-Paired, Saline-Paired) mixed ANOVA confirmed a 4-way interaction $F(2,48) = 4.27$, $p = .02$. Generally, the rats engaged in considerable exploratory behaviour often crossing between chambers, but as time passed rats explored less and spent more time on the morphine-paired side. This can be seen in the black-paired and white-

Morphine-induced CPP in HIV-1Tg rats

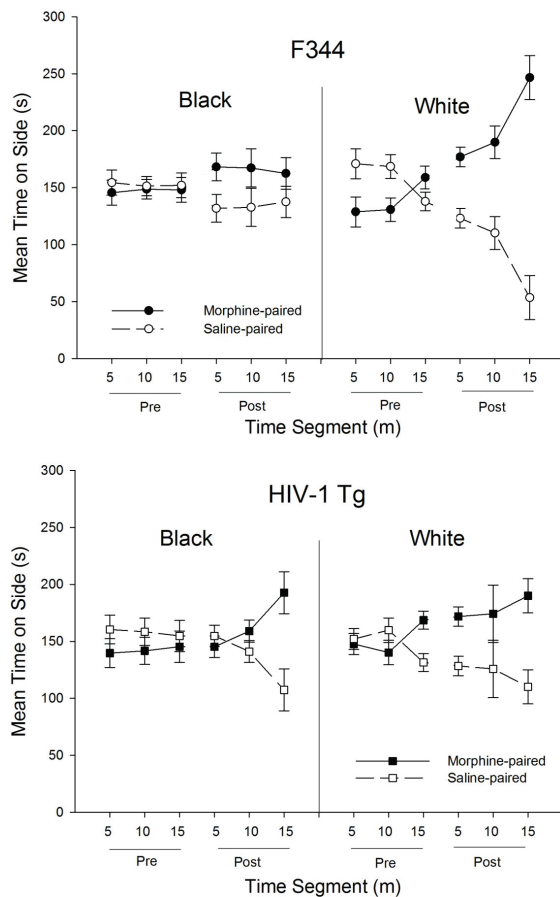


Figure 3. Time spent in the morphine-paired and saline-paired side of the CPP apparatus by strain and by color of morphine-paired chamber separated into three 5-minute time segments, in pre-conditioning and post-conditioning phase is shown.

paired HIV-1Tg groups (bottom graph). Although this pattern of increasing preference over time was not seen in the black-paired F344 group it was very pronounced in the white-paired F344 rats (top graph). When the animals were first placed in the CPP apparatus they explored and might have spent more time exploring in the preferred sides, but as the exploratory behaviour habituates the time spent in the preferred side increases even more. The data suggests that the F344 rats that experienced morphine paired with the white side had the strongest preference for the morphine-paired side (Figure 3).

Extinction

To test for extinction (no difference in time spent in the morphine-paired side and saline-paired side) animals were given free access to

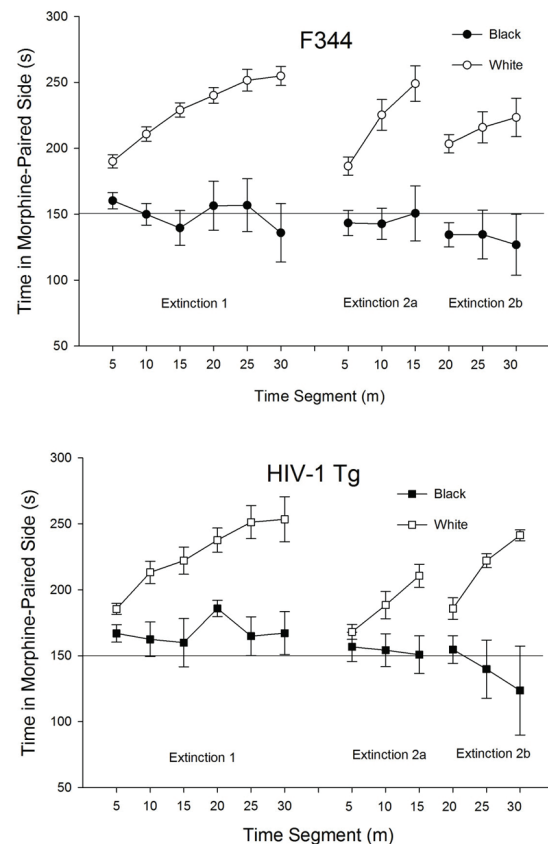


Figure 4. Extinction phase: To test for extinction, animals [F344 (n=14), HIV-1Tg (n=14)] were given free access to the entire apparatus for 30 mins (F344: 8 days, HIV-1Tg: 8 days) every day (Extinction 1) and then for 2 fifteen min sessions/day (F344: 9 days, HIV-1Tg: 13 days) (Extinction 2A and 2B) until all animals reach extinction. Average time spent in the morphine-paired side by strain and by color of morphine-paired chamber separated into three 5-minute time segments (group means) in the 2 phases of extinction [Extinction 1 and Extinction 2 (A and B)] is shown.

the both chambers for 30 minutes per day for 8 days (Extinction 1) and for 2 sessions of 15 minutes per day thereafter (Extinction 2A and 2B).

The time spent in the morphine-paired side in 5-minute time segments for two phases of extinction is shown in Figure 4. A score of 150 indicates equal time spent in the black and white chambers per time segment (no preference); a score greater than 150 indicates a preference for the morphine-paired side. The average across all days for each extinction phase is plotted in order to show the high resistance to extinction in the rats that experienced morphine

paired with the white chamber and to demonstrate the observed within-session changes in preference. We hypothesize that the within-session increase in preference is due to habituation of exploratory behaviour in the presence of a highly preferred white chamber. Evidence consistent with this habituation interpretation is provided in Extinction 2 where spontaneous recovery of exploratory behaviour was observed as a result of the delay between Extinction 2A and 2B (Figure 4). These results suggest that the rats had a natural preference for the white side that was not evident in the pre-conditioning phase, but when paired with morphine it became a much stronger preference that was highly resistant to extinction.

If habituation of exploratory behaviour contributes to the increasing preference over time in the CPP test, as hypothesized here, then the preference at the beginning of Extinction 2B (minutes 16-20) should be less than the preference observed at the end of Extinction 2A (minutes 10-15) because the delay causes the spontaneous recovery of exploratory behaviour. This is the pattern seen in the Extinction 2 data of the white-morphine paired groups that are plotted in Figure 4. Note that this drop in preference between minutes 10-15 and minutes 16-20 is not seen in Extinction 1 since there was no delay imposed between these time segments to allow for spontaneous recovery of exploratory behaviour. To confirm these observations a spontaneous recovery score was calculated for all rats by subtracting the time spent on the morphine-paired side during the fourth time segment (minutes 16-20) from the time spent on the morphine-paired side during third time segment (minutes 11-15). A positive score indicates spontaneous recovery, whereas a negative score indicates continued habituation. To facilitate statistical analysis of Extinction 1 (which had 8 days) and Extinction 2 (which had 9 days), the last day of extinction 2 was omitted. The data was then analyzed with a Strain (2) x Morphine Color (2) x Extinction Phase (2) by Days (8) mixed ANOVA. The white-morphine paired and black-morphine paired HIV-1Tg and F344 groups averaged across days for the two extinction sessions are shown in Figure 5. Spontaneous recovery was observed in the white-morphine paired groups (right panel) in Extinction 2, when a delay was present, but not in Extinction 1 when there was no delay. These observations were supported by a significant

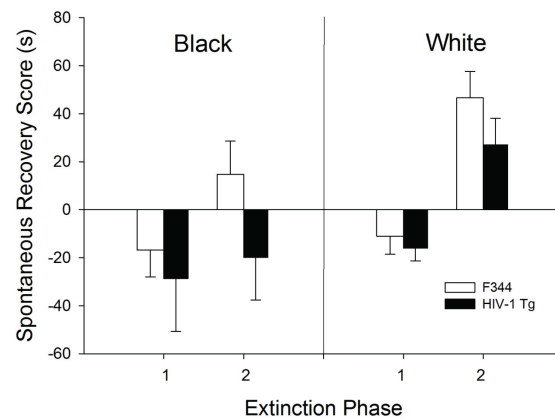


Figure 5. Spontaneous Recovery Score- The time spent on the morphine-paired side during the fourth time segment (minutes 16-20) was subtracted from the time spent on the morphine-paired side during third time segment (minutes 11-15). A positive score indicates spontaneous recovery, whereas a negative score indicates continued habituation. The results for the white-morphine paired and black-morphine paired HIV-1 and F344 groups averaged across days for the two extinction sessions are shown. To facilitate statistical analysis of Extinction 1 (which had 8 days) and Extinction 2 (which had 9 days), the last day of extinction 2 was omitted.

main effect of Extinction Phase, $F(1,24) = 17.3$, $p = .000$. A significant main effect of color of morphine-paired chamber, $F(1,24) = 6.14$, $p = .021$, supports the impression that spontaneous recovery was greater in the white-morphine paired groups than the black-morphine paired groups. There was no significant effect of days. The results also suggest that spontaneous recovery was greater in the F344 rats than the HIV-1Tg rats, but the main effect of strain fell short of significance, $F(1, 24) = 3.29$, $p = .08$ and strain did not interact with any other factor.

These pattern of data suggest that during a CPP test there are several factors that influence overall behaviour: a conditioned place preference, a natural preference (when present), and the elicitation of exploratory behaviour that interacts with the conditioned and natural preferences. The impact of the two preferences on performance differs across a session as exploratory behaviour undergoes habituation, with the stronger preference dominating control of behaviour later in a session as the rats begin to "settle into" one chamber. Whereas a strong natural preference for one side may obscure a more modest conditioned place preference later

F344 / Morphine Paired with White Side

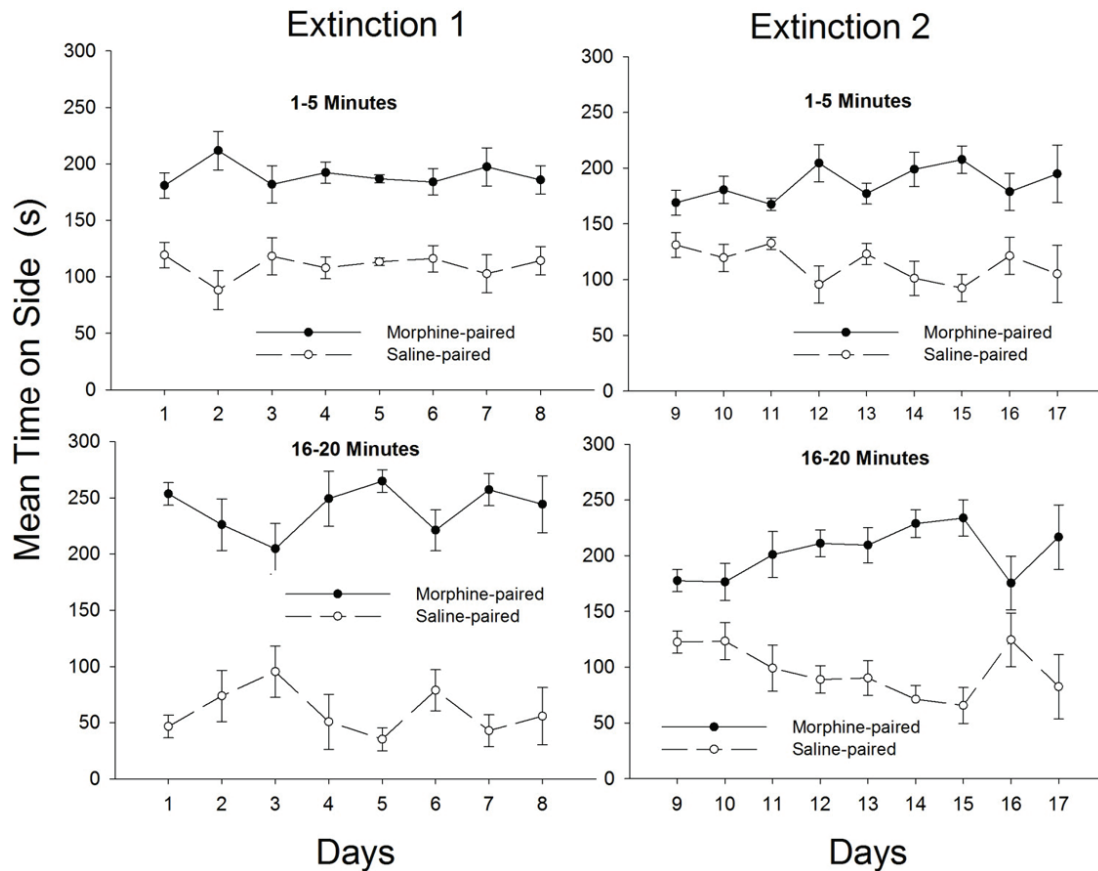


Figure 6. The time spent in the morphine- and saline-paired sides over days of the F344 animals that received morphine paired with the white side. The Extinction 1 graphs are shown on the left side and Extinction 2 are shown on the right. Data from only 2 of the 6 time segments are shown. The top graphs show the first time segment (minutes 1-5) and the lower graphs show the fourth time segment (minutes 16-20). During Extinction 2 the 16-20 minute time segment was preceded by a break from the previous session to allow for spontaneous recovery of exploratory behavior.

in a session, the CPP may have a greater contributing role in overall performance earlier in a session when exploratory behaviour is at its maximum. We suggest that the strong preference for the white chamber in the rats that received the white side paired with morphine is a result of an enhanced natural preference for the white side rather than a result of the summation of a conditioned place preference with a natural preference. If the latter was true, then preference in the first 5 minutes of an extinction session (when a modest CPP is having its greatest impact on performance) should decline over days as the CPP undergoes extinction, whereas the preference later in a session (which reflects the stronger natural preference) should persist

over days. However, if morphine induced the enhancement of a natural preference that is resistant to extinction, then the observed preference should be seen at all time intervals across all days with no evidence of extinction. The time in the morphine- and saline-paired sides of the F344 animals that received morphine paired with the white side is shown over days for two of the six 5-minute time segments in **Figure 6**. A preference for the morphine-paired side in the first 5 minutes (top panels) is observed that does not change over days in Extinction 1, [Effect of Drug: $F(1,5) = 63.62$, $p = .000$; Drug \times Days: $F(7, 35) < 1$, n.s.], or in Extinction 2 [Effect of Drug: $F(1,5) = 28.27$, $p = .003$; Drug \times Days: $F(8, 40) = 1.34$, $p = .251$]. Thus, there is

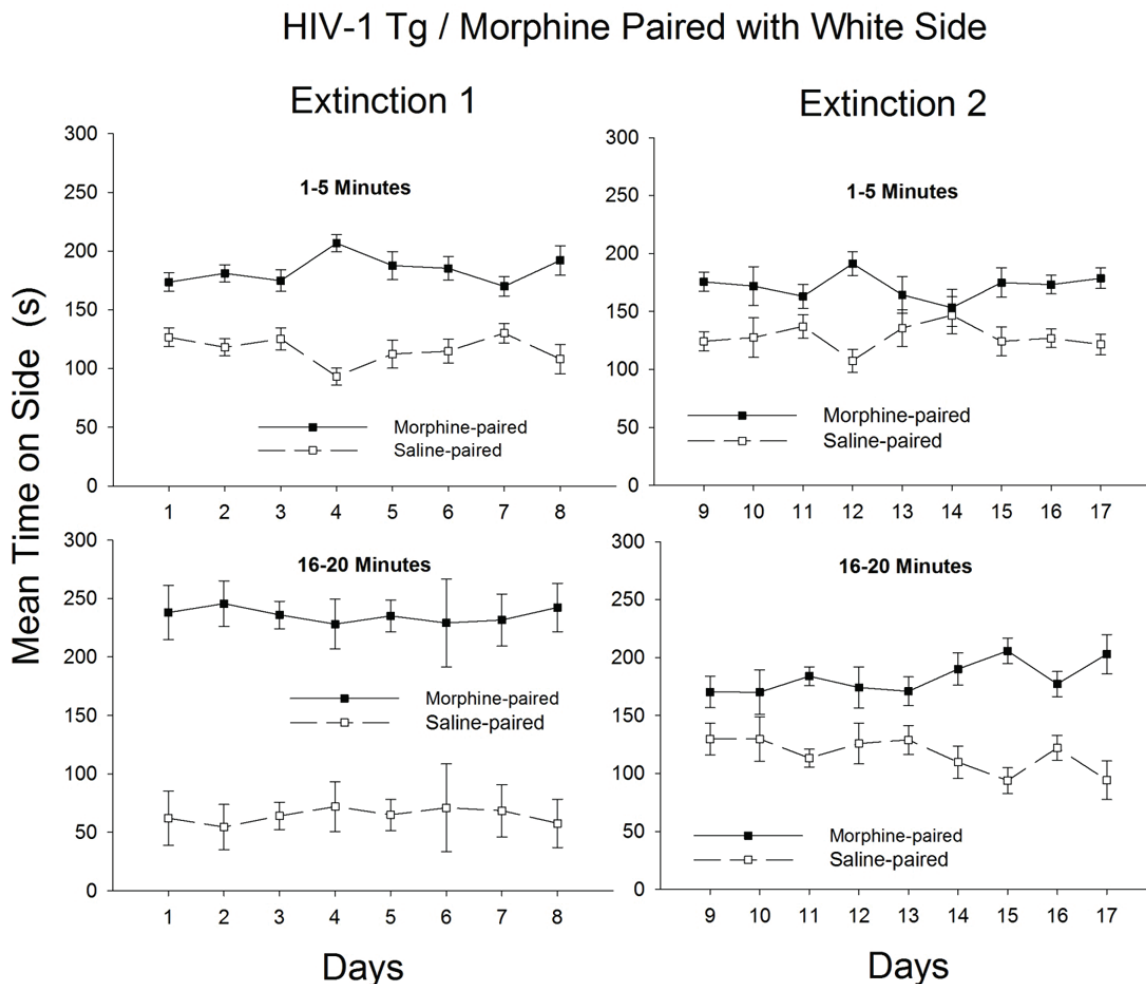


Figure 7. The time spent in the morphine- and saline-paired sides over days of the HIV-1Tg animals that received morphine paired with the white side. The Extinction 1 graphs are shown on the left side and Extinction 2 are shown on the right. Data from only 2 of the 6 time segments are shown. The top graphs show the first time segment (minutes 1-5) and the lower graphs show the fourth time segment (minutes 16-20). During Extinction 2 the 16-20 minute time segment was preceded by a break from the previous session to allow for spontaneous recovery of exploratory behaviour.

no evidence of extinction over days, even when just the first 5 minutes, when a CPP is likely to have its greatest impact, are examined. By the fourth time segment (minutes 16-20) of Extinction 1 the preference for the morphine-paired time is even greater (bottom, left panel) as exploratory behaviour begins to habituate. However, during Extinction 2, when the 16-20 minute time segment was preceded by a break, the preference is again reduced as exploratory behaviour spontaneously recovers. Yet there is still no indication of a decreased preference for the morphine-paired side over days, [Drug x Days: $F(8, 40) = 1.73$, $p = .121$]. The same pattern is also seen in the HIV-1Tg rats (**Figure 7**).

The pattern of results for the rats that received morphine paired with the black side was very different. **Figures 8 and 9** show the data for the F344 and the HIV-1Tg rats respectively. These were plotted in the same manner as the previous graphs. The Extinction 1 and 2 data were analysed separately with a Strain (2) x Drug (2) x Days (8 or 9) mixed ANOVA. During the first 5 minutes of Extinction 1 there was a significant preference for the morphine-paired side that persisted across days of Extinction 1 in both strains [Main Effect of Drug: $F(1,12) = 6.32$, $p = .027$; Drug x Days: $F(7,84) < 1$, n.s.]. There were no significant effects of strain. By Extinction 2 the main effect of Drug was no longer

F344 / Morphine Paired with Black Side

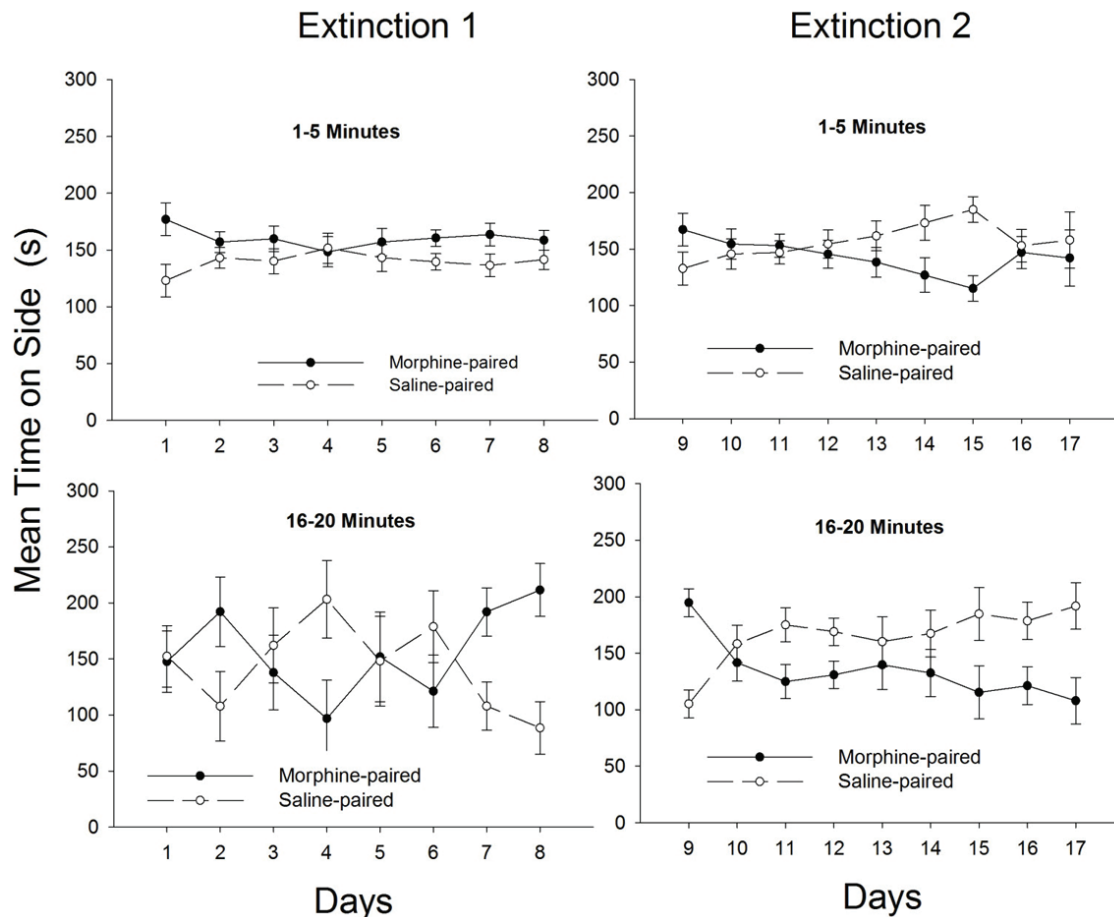


Figure 8. The time spent in the morphine- and saline-paired sides over days of the F344 animals that received morphine paired with the black side. The Extinction 1 graphs are shown on the left side and Extinction 2 are shown on the right. Data from only 2 of the 6 time segments are shown. The top graphs show the first time segment (minutes 1-5) and the lower graphs show the fourth time segment (minutes 16-20). During Extinction 2 the 16-20 minute time segment was preceded by a break from the previous session to allow for spontaneous recovery of exploratory behaviour.

significant [$F(1,12) < 1$, n.s.] indicating the extinction of a CPP. Once again there were no significant effects of strain.

During the fourth time segment (minutes 16-20) a reversal in preference from the black morphine-paired side to the white saline-paired side took place during Extinction 2 in the F344 rats (**Figure 8**) [$F(7, 49) = 2.41$, $p = .034$]. The absence of a significant preference during the 16-20 minute time segment of Extinction 1 may reflect the persistence of a conditioned preference for the black chamber that competed with the natural preference for the white chamber.

By Extinction 2 the extinguished CPP no longer competed with the natural preference, reversing the side preference. This reversal during minutes 16-20 in Extinction 2 was not observed in the HIV-1Tg rats [$F(7,35) < 1$, n.s.] (**Figure 9**), however, suggesting that CPP was not fully extinguished in the HIV-1Tg rats. Thus, the HIV-1Tg rats were tested for 3 additional days for extinction.

Figure 10 shows the results of the last three extinction days for the HIV-1Tg rats. There was no effect of days; therefore the means across all three days are presented. A significant four-

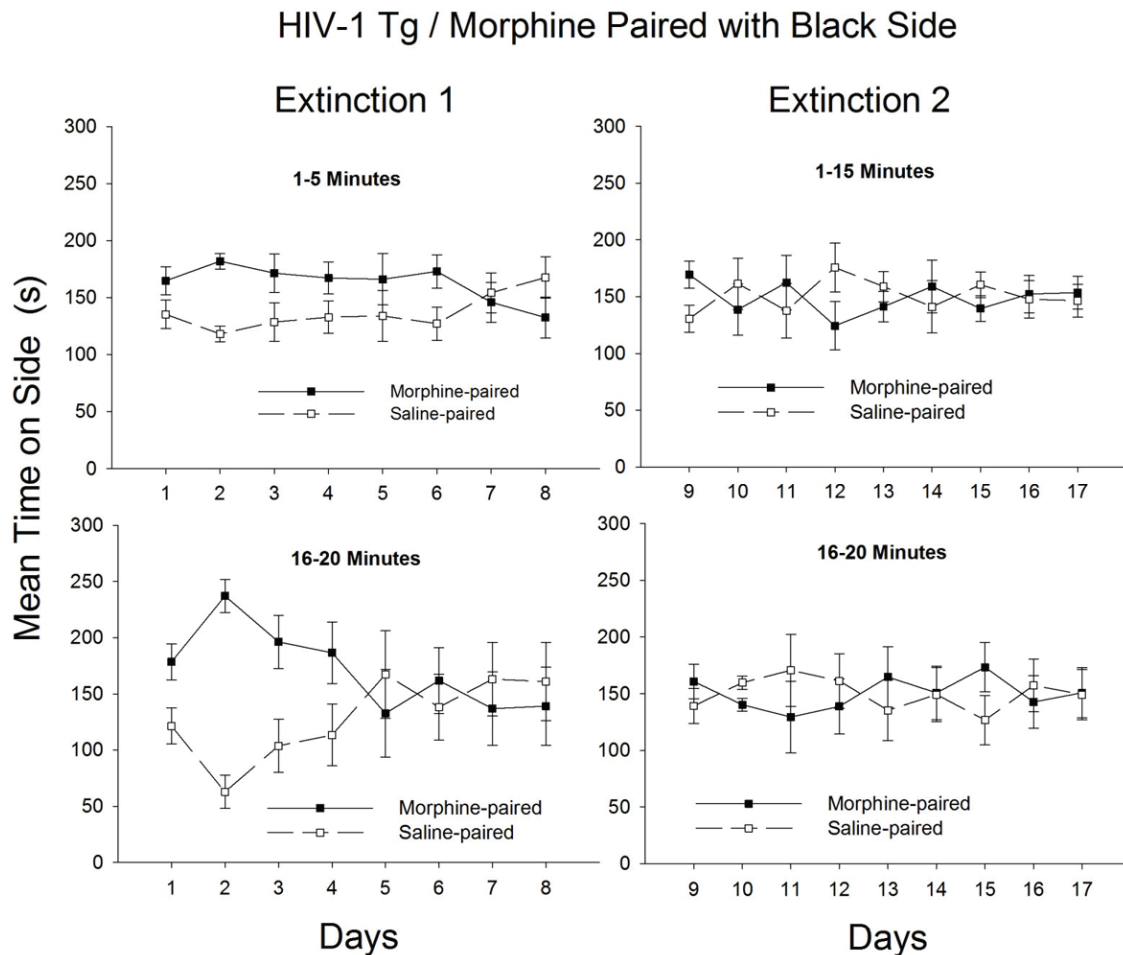


Figure 9. The time spent in the morphine- and saline-paired sides over days of the HIV-1Tg animals that received morphine paired with the black side. The Extinction 1 graphs are shown on the left side and Extinction 2 are shown on the right. Data from only 2 of the 6 time segments are shown. The top graphs show the first time segment (minutes 1-5) and the lower graphs show the fourth time segment (minutes 16-20). During Extinction 2 the 16-20 minute time segment was preceded by a break from the previous session to allow for spontaneous recovery of exploratory behaviour.

way interaction was found $F(4,48) = 3.39$, $p = .016$. The rats for whom morphine was paired with the white side (top graph) continued to show a significant preference for the morphine-paired side at all time segments and the preference increased across the first session as well as across the second session after spontaneous recovery of exploratory behaviour. The rats for whom morphine was paired with the black side (lower graph) did not show a preference during the first time segments of both extinction sessions (time segments 1-5 and 16-20) but showed a reverse preference in the subsequent sessions. Thus, although a reverse preference was not observed in the first five minutes of

extinction like in the F344 rats, the strong preference for the saline-paired side later in the sessions is consistent with the suggestion that the white chamber is a highly preferred chamber that was not evident in preconditioning test.

Reinstatement with re-introduction of morphine

F344 ($n=7$) and HIV-1Tg ($n=7$) after reaching the extinction state were given 1mg/kg morphine and tested for CPP by giving them free access to the entire apparatus for 15 minutes. On the next day the same animals were given a higher dose of morphine (3.5 mg/kg, i.p.) and then free access to the entire apparatus for 15

Morphine-induced CPP in HIV-1Tg rats

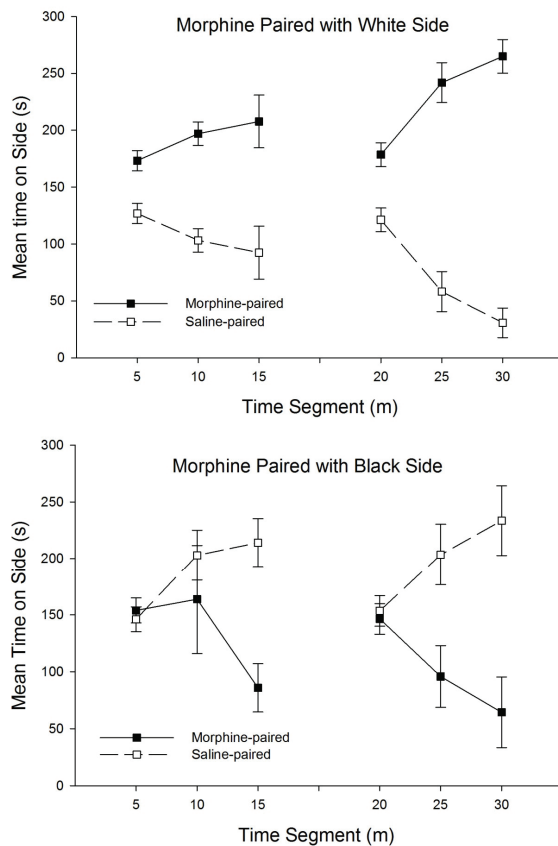


Figure 10. Time in Morphine-paired chamber over time segments for HIV-1Tg rats given three additional days of extinction during the second extinction phase (Mean of Days 18-20).

minutes. The HIV-1Tg and F344 animals that had morphine paired with the black side showed a preference for the saline-paired side during the reinstatement test. This supports the argument that the rats had a natural preference for the white side that was counteracted by the CPP, but when the CPP is extinguished the animals chose to spend more time on the white side during all three time segments despite the reinstatement attempt. The data from the HIV-1Tg and F344 rats that had the morphine paired with the white side confirms that they had a preference for the white side. It cannot be assumed that this preference is a reinstatement effect because they never extinguished this preference during the extinction phase.

Reinstatement with a foot shock stressor

After reaching extinction the F344 (n=7) and HIV-1Tg (n=7) were administered a foot-shock for 15 min. intermittently and the tested for CPP

by giving them free access to the entire apparatus for 15 min. The F344 and HIV-1Tg rats that had the morphine paired with the white side showed a preference for the white chamber that increased across the time segments (Tim Seg x Drug: F344, $F(2,4) = 29.245$, $p = .004$; HIV-1Tg, $F(2, 6) = 7.773$, $p = .022$). These groups, of course, cannot be used to assess reinstatement because their preference for white never extinguished, but they confirm that the white preference in the white-morphine paired groups observed throughout extinction was still present. However, no statistically significant effects were observed in the F344 or HIV-1Tg rats that had morphine paired with the black side.

Discussion

In the present study, we explored the hypothesis that the HIV-1Tg rat is more prone to the addictive properties of morphine than the control animal F344 because expression of MOR is up-regulated in the HIV-1Tg rat [9]. We used the CPP test to measure the motivational salience of morphine paired cues of the HIV-1Tg rats versus control, F344 rats. A dose of 3.5 mg/kg morphine successfully established a CPP in both the HIV-1Tg and F344 rats. However, an unanticipated finding was that rats that had morphine paired with the white chamber failed to show any evidence of extinction, whereas the rats that had morphine paired with the black chamber showed successful extinction and the emergence of a reversed preference. When the color of the morphine-paired chamber was not taken into consideration in the statistical analysis, group averages suggested that extinction occurred after 17 and 20 days for F344 and HIV-1Tg rats, respectively. However, when the morphine-paired chamber was included as a factor in the analysis, it was revealed that extinction occurred earlier in the rats for whom morphine was paired with the black chamber, but not in the rats for whom morphine was paired with the white chamber.

During a CPP test rats freely explore both sides of the apparatus, spending more time overall on the morphine-paired side if a CPP is established. The behaviour of the rats in a CPP apparatus, however, is affected by factors other than a CPP that are not typically assessed systematically in CPP studies. When first placed in the apparatus the relative novelty of the environment activates exploratory behaviour that declines or habituates over time. As exploratory

behaviour undergoes habituation the rats move less and choose to settle in a preferred chamber if a preference is present [36]. Rats may settle in one chamber because that chamber accrued rewarding properties when it was paired with morphine; this is basic assumption of the CPP test. However, our present studies suggest that following habituation of exploratory behaviour rats may also settle to one chamber when a natural preference for that chamber exists that may not have been apparent in the preconditioning phase when the novelty of the apparatus was activating high levels of exploratory behaviour.

The presence or absence of natural preferences is a characteristic of the CPP apparatus that has been considered in the literature. When morphine is paired with the naturally preferred chamber, the observed CPP is assumed to result from the sum of the natural preference and the conditioned preference. When morphine is paired with the non-preferred chamber, however, the conditioned rewarding properties of the previously less preferred chamber will compete with the naturally preferred chamber. If the newly acquired rewarding properties are sufficiently strong to compete successfully with the natural preference a CPP will be observed, although it is likely to be weaker than in subjects that had morphine paired with the naturally preferred side. This is the reason that the drug is paired with the non-preferred side when a biased CPP apparatus is used [34]. A successful CPP in a biased apparatus indicates that the conditioned rewarding properties were sufficiently strong to overcome the natural preference. With an unbiased apparatus the drug is not paired with a preferred side although the drug-paired chamber is typically counterbalanced within groups. But the results of the present study suggest that natural chamber preferences may not be apparent when a single preconditioning test is used, a common procedure in the CPP literature. This is because that there is not sufficient opportunity for the habituation of intense exploratory behaviour to reveal the natural preference. When we analyzed the preconditioning and post-conditioning results separated into three 5-minute time segments no statistically significant side preference was observed during preconditioning in the two-chamber CPP apparatus used in the present experiment. However, the post-conditioning data showed clear changes in preference over

the three 5-minute periods. As time passed rats generally explored less and spent more time in the morphine-paired chamber. This pattern was most pronounced in the rats that experienced morphine paired with the white chamber.

There were animals that did not extinguish the morphine-induced CPP and there were other animals that did show extinction. For clarity these are discussed separately below.

Failed extinction of a CPP

The extinction data indicated that the preference for the white morphine-paired side failed to weaken despite 17 (F344 rats) and 20 (HIV-1 Tg rats) days of exposure to the extinction procedure. Moreover, within-session increases in preference for the morphine-paired side was consistently observed in the white-morphine paired rats, but not in the black-morphine paired rats. We argue that the within-session increase in preference is due to habituation of exploratory behaviour. Evidence consistent with this habituation interpretation is provided in Extinction 2 where spontaneous recovery of exploratory behaviour was observed as a result of the delay between Extinction 2A and 2B. Our studies also show that the rats had a natural preference for the white chamber that was not evident in the preconditioning phase, but when paired with morphine this preference became more evident. The natural place preference plus the conditioned place preference may have synergized to augment the preference for the white side. An alternative explanation is that morphine enhanced the natural preference without inducing a CPP as it is typically characterized.

If the increased preference for the white chamber was due to the summing of a natural preference and a CPP, then repeated exposure to the chamber cues in the absence of morphine treatment should decrease the place preference as the CPP undergoes extinction, but there was no evidence of extinction in the white-morphine paired groups. It cannot be ruled out that extinction would have been observed if additional extinction days were given. However, the expectation of an increased likelihood of extinction given sufficient extinction trials is based on the prevailing theoretical view that explains CPP as a case of classical conditioning that involves predictive learning. Alternatively, the CPP procedure with morphine may result in another form

of behavioural plasticity that does not conform to a predictive learning account of classical conditioning.

In the predictive learning model of classical conditioning the acquisition of CPP is seen as involving an association between the discriminative chamber cues and the rewarding properties of morphine, such that the chamber cues predict the availability of morphine. In the extinction procedure a new association is presumed to occur (i.e., the chamber cues no longer predict the availability of morphine) that supersedes the original association, causing CPP to extinguish. When the rats are "reminded" of the earlier predicative relationship in a reinstatement procedure the CPP reemerges; that is, the morphine effect is once again anticipated. However, there are several well documented cases of classical conditioning procedures that result in a conditioned preference that do not extinguish. When the consumption of a flavored saccharin solution is paired with a carbohydrate solution that is pumped directly into the stomach, for example, rats develop a strong preference for the flavored saccharin solution that is highly resistant to extinction [37, 38]. This suggests that some preferences that are established with classical conditioning procedures may not be easily eliminated by extinction. A body of research has found similar effects in animals and humans and is referred to as evaluative conditioning [39, 40]. In evaluative conditioning the positive value of the unconditioned stimulus is presumed to transfer to the conditioned stimulus, increasing the liking of the conditioned stimulus. Therefore, the positive value of the morphine unconditioned stimulus in the present study may have been transferred to preferential discriminative chamber cues (white chamber) to produce an enhanced preference that is highly resistant to extinction. Indeed a persistent preference for cues previously paired with a psychoactive drug would help explain an individual's life-time struggle with drug addiction - the acquired preference for drug-paired cues never really go away. Interestingly, numerous studies show that conditioned responses to drug-paired cues can undergo extinction in animals, but the evidence of similar extinction effects in humans is lacking [41].

It is unclear what factors may be involved in whether a CPP undergoes extinction or whether it persists indefinitely. One possible factor that

may influence resistance to extinction may be the type of morphine-paired cue. A CPP that resists extinction may be more likely if there is a natural preference for the paired cue before conditioning, even if the natural preference is not immediately apparent early in testing because other behaviours (e.g., exploratory behaviour) mask the initial preference. Clearly not all preferred cues paired with a drug lead to CPP that fails to extinguish since extinction is often observed in biased CPP procedures where the drug is paired with an initially preferred side. An enhanced natural preference may be most prominent when morphine is paired with contact cues (that is, floor cues) because once rats make physical contact with the floor cues they may prefer to maintain contact with these cues and reduce their approach to other distal cues [42].

The prevalence of CPP that is highly resistant to extinct is not well known. Investigators often do not test for extinction of CPP because it is not germane to the hypothesis being investigated. When extinction is a desired outcome in an experiment (e.g., to test for reinstatement, for example) any procedure that results in CPP that resists extinction may be interpreted as a failed procedure and abandoned in favor of procedures that yield CPP results more consistent with a predictive memory model interpretation. Nevertheless there are several examples of high resistance to extinction of morphine-induced CPP in the literature. Most recently, for example, Rutten and colleagues reported that it took 18 fifteen-minute-sessions to see extinction of a 3.0 mg/kg dose and up to 45 fifteen-minute-sessions for a 10 mg/kg dose of morphine [25]. In another recent study, Narp knockout mice failed to extinguish a morphine CPP. The authors conclude that the Narp deletion disrupts the ability to update the predictive association between the CPP context cues and the drug reward [43]. However, this conclusion assumes that morphine CPP procedures result in associative predictive learning. If CPP can also result in the establishment of long lasting preference that is not subsequently altered through extinction, then it is difficult to determine if Narp deletion failed the updating of predictive learning (failed extinction) or if it resulted in an enhanced behavioural plasticity that is better described as evaluative conditioning. It is also possible that a preference for floor cues may have been altered in the transgenic mice, rather than

their propensity to learn, and morphine induced an enhancement of this altered, natural preference. We suggest here that the interpretation of extinction performance of morphine CPP may be complicated by the behavioural plasticity noted in the present CPP experiment and a more detailed analysis of changing preferences across CPP sessions can help evaluate the factors that contribute to the acquisition and maintenance of a place preference.

It is still plausible that a CPP was established along with an enhanced natural preference in the white morphine paired animals, but the natural preference dominated over the CPP. Thus, we examined place preference early in an extinction session (minutes 1-5), when the animals are exploring at their maximum because habituation has not yet occurred. The exploratory behaviour early in the session may be guided primarily by the morphine predictive cues (i.e., CPP) independent of a natural preference. The same can be said for minutes 16-20 in Extinction 2B, because the delay allowed the spontaneous recovery of exploratory behaviour in this time segment.

A preference for the morphine-paired side in the first 5 minutes was observed that did not decrease over days in Extinction 1 or Extinction 2. Thus, there was no evidence of extinction of morphine-predictive cues over days, even when just the first 5 minutes are examined. By the fourth time segment (minutes 16-20) of Extinction 1 the preference for the morphine-paired time was even greater as exploratory behaviour was habituating, but during Extinction 2 the preference is again reduced as exploratory behaviour spontaneously recovers. Yet there is still no indication of a decreased preference for the morphine-paired side over days. The same pattern was also seen in the HIV-1Tg rats. The evidence suggests, therefore, that morphine induced enhanced natural preference when it was paired with the white chamber rather than a CPP. Perhaps this enhanced natural preference is best described as a type of evaluative conditioning [39, 40]. A limitation of the evaluation conditioning hypothesis is that a control group that received no morphine treatment but similar number of "extinction" exposures to the chamber was not available to assess the strength of the natural preference in the absence of morphine. Nevertheless, the evaluative conditioning hypothesis is worth exploring given the many

cases in the literature of preferences that are highly resistant to extinction.

Extinction of a CPP

The same analysis of the data over time segments from the black-morphine paired groups yielded evidence of the extinction of a CPP. The evidence suggests that these groups established a CPP that overcame the natural preference for the white chamber and that this CPP extinguished. Although the black-morphine paired groups did not experience an enhancement of the natural preference for the white chamber (because it was not paired with morphine) the hypothesized natural preference for the white side should still be present. As extinction of the CPP occurs the natural preference for the white side should emerge. This is what was observed.

During the first 5 minutes of Extinction 1 there was a significant preference for the morphine-paired side that persisted across days of Extinction 1 in both the F344 and HIV-1Tg groups. By Extinction 2 the main effect of drug was no longer evident, indicating that the CPP had extinguished.

During the fourth time segment (minutes 16-20) of Extinction 2A significant reversal in preference from the black morphine-paired chamber to the white saline-paired chamber was observed in the F344 rats. This preference reversal from the black morphine-paired chamber to the white saline-paired chamber during minutes 16-20 in Extinction 2 was not observed in the HIV-1Tg rats, however, suggesting that CPP was not fully extinguished in the HIV-1Tg rats. An additional 3 days of extinction for the HIV-1Tg rats still failed to result in a reverse preference during the first 5 minutes of the extinction sessions, but the reverse preference was clearly evident in the last 10 minutes of the extinction sessions.

The lack of preference reversal in the HIV-1Tg rats during the first 5 minutes of extinction suggests that the HIV-1Tg rats showed greater resistance to extinction of a CPP than the F344 rats. A more substantial resistance to extinction was observed in the HIV-1Tg rats in a previous study [44], nevertheless both studies support the hypothesis that morphine has a greater rewarding efficacy in the HIV-1Tg rats [25]. The

difference in the magnitude of the resistance to extinction that was observed between the two studies is most likely due to the apparatus used. In the previous study a custom-made three-compartment apparatus was used that resulted in a very strong natural preference for the smaller middle compartment. Many rats also showed a slight preference for one of the two larger compartments. Morphine was paired with the less preferred of the two larger chambers while saline was paired with the other large chamber. The middle chamber was not paired with any injection. To assess CPP the time in the two larger chambers were compared while the time in the middle chamber was not taken into consideration. The rats spent most of their time in the naturally preferred chamber, but when the times in the two larger chambers were compared a significant CPP was observed that took significantly longer to extinguish in the HIV-1Tg rats. Thus, in the previous study with a three-compartment chamber the strong natural preference for the smaller chamber did not compete directly with the CPP that was established in the larger chambers, whereas in the current study with the two-compartment apparatus the natural preference competed with the CPP.

Reinstatement of CPP

A salient feature of drug addiction is relapse [19, 20]. Even after long drug-free periods the subject can relapse to drug seeking and use if exposed to small quantities of the drug, to a drug-paired cue or to stress [20, 45]. CPP has been used to measure reinstatement for extinguished place preference by reintroduction of the drug (drug-priming) or to stressors [14, 30, 46-48].

In the present experiment we used morphine primes of 1 mg/kg and 3.5 mg/kg to reinstate the CPP. The HIV-1 TG and F344 rats, however, failed to show a drug prime reinstatement effect. The HIV-1Tg and F344 rats for which morphine was paired with the black chamber preferred the saline-paired side during the reinstatement test. This supports the argument that the rats had a natural preference for the white side that was counteracted by the CPP, but after the CPP extinguished they chose to spend more time on the white side despite the reinstatement effect. It may be that this white side preference was overcome by the initial CPP, but the reinstatement effect was just not strong

enough to overcome this natural preference.

There is considerable research indicating that there are major differences in the neurocircuitry and neurobiology of reinstatement by drug-priming, by cues and by stressors [47, 49]. Stewart and colleagues show that the corticotrophin-releasing factor and noradrenergic dependent pathways were primarily involved in foot-shock stress induced relapse but had little effect on drug-induced relapse [50-53]. There are studies which indicate that stress is a co-risk-factor in the drug-addiction relapse in the HIV-1 positive population [54-57]. Under foot shock stress the F344 and HIV-1Tg rats that had the morphine paired with the white side continued to show a preference for the white side. However, no statistically significant effects were observed in the F344 or HIV-1Tg rats that had morphine paired with the black side. This result may suggest that the stress reinstated a CPP sufficient enough to counteract the preference for white, but not enough to reverse the preference for the morphine-paired side. An alternative explanation is that the black-morphine paired animals were showing a smaller preference for the white side by the end of extinction (the natural white side preference was not enhanced for them because it was never paired with morphine) and in these reinstatement tests the smaller preference is no longer evident because of the reduced statistical power as a result of reducing the number of subjects by about half.

The failure to observe reinstatement effects in this experiment precluded the test of the hypothesis that HIV-1Tg and F344 rats differ in their sensitivity to morphine. There was some evidence, however, that the HIV-1Tg rats display a greater resistance to extinction of a morphine CPP; a result that is consistent with a previous experiment using a three-compartment biased CPP apparatus [14]. These differences in resistance to extinction of a morphine CPP supports the hypothesis that viral proteins alters the effects of morphine on behaviour, but more studies and a better understanding of the behavioural plasticity that occurs in the morphine CPP procedure is needed before these effects of viral proteins can be elucidated.

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