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Involvement of κ/Dynorphin System in WIN 55,212-2 Self-Administration in Mice

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Self-administration procedures have not yet provided evidence that freely moving mice can reliably acquire and maintain an operant behavior to self-administer cannabinoid agonists. The aim of the present work was to establish a model of cannabinoid operant intravenous self-administration in freely moving mice given the relevance of this species for the use of genetically modified animals. In addition, the possible involvement of the κ/dynorphin system in cannabinoid self-administration was evaluated by using pro-dynorphin knockout mice. Outbred CD1 wild-type mice as well as pro-dynorphin knockout and wild-type mice were trained to self-administer the cannabinoid receptor agonist WIN 55,212-2 under an FR1 schedule of reinforcement. Two cannabinoid training doses (6.25 and 12.5 μg/kg/infusion) were used in the acquisition studies in outbred mice. Animals acquired a reliable operant responding to self-administer WIN 55,212-2 (12.5 μg/kg/infusion), but required as many as 15 sessions to attain this behavior. Interestingly, when a previous injection of WIN 55,212-2 (0.1 mg/kg, i.p.) was administered in the home-cage 24 h before the first session, mice acquired operant responding for cannabinoid self-administration by the fourth session. When the κ-opioid agonist antagonist nor-binaltorphimine (5 mg/kg s.c.) was administered 4 h before the first session, the time required to acquire a reliable cannabinoid self-administration was also significantly reduced. Finally, a shift to the left in the dose-intake curve to self-administer WIN 55,212-2 was observed in pro-dynorphin knockout mice when compared to wild-type mice. These results indicate that the activation of the κ/dynorphin opioid system after WIN 55,212-2 administration could counteract cannabinoid rewarding effects.
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INTRODUCTION

Cannabis sativa derivatives, such as marijuana and hashish, are the most widely consumed illicit drugs (Smart and Ogborne, 2000), and produce clear addictive effects in humans. Whereas most recreational users of cannabis experience a state of euphoria, others also report dysphoria and anxiety particularly at the beginning of cannabis consumption (Greg *et al*, 1976; Thomas, 1993; Grinspoon and Bakalar, 1997; Williamson and Evans, 2000). Human reports are in agreement with animal studies showing that Δ^9-tetrahydrocannabinol (THC) and other synthetic cannabinoid agonists can induce both rewarding and aversive effects, as revealed in the place conditioned paradigm (Sañudo-Peña *et al*, 1997; Cheer *et al*, 2000; Valjent and Maldonado, 2000). The endogenous opioid system seems to play an important role in the different effects produced on the reward circuit after cannabinoid administration. Accordingly, both pharmacological and genetic studies have provided evidence that the rewarding effects of cannabinoids are mediated by the activation of μ-opioid receptors, whereas the stimulation of κ-opioid receptors (KORs) by opioid peptides derived from pro-dynorphin seem to mediate their aversive effects (Ghozland *et al*, 2002; Zimmer *et al*, 2001). Moreover, several studies suggest that the κ/dynorphin system opposes drug-rewarding effects, giving support to the idea that KORs could act as a possible pharmacotherapy for drug dependence (for a review, see Hasebe *et al*, 2004). Thus, KORs inhibit the rewarding effects of several drugs of abuse, such as morphine or cocaine, and also attenuate the elevation of dopamine turnover induced by morphine in the limbic forebrain (Narita *et al*, 1993; Schenk *et al*, 1999).

Cannabinoids, such as THC, can stimulate the release of the endogenous KOR agonist dynorphin (Welch and Eads, 1999; Houser *et al*, 2000), which may contribute to their initial aversive effects. These dysphoric effects induced by the first administration of cannabinoids in rodents seem to be crucial to mask their rewarding properties in behavioral models. Thus, conditioned place preference induced by

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THC or WIN 55,212-2 (WIN) can only be revealed in mice if they received a previous injection of the drug 24 h before the first conditioning day (Valjent and Maldonado, 2000; Castañé *et al*, 2004). Studies with genetically modified animals have provided further support to this hypothesis and have revealed the specific involvement of the κ/dynorphin system in such an initial aversive response of cannabinoids (Zimmer *et al*, 2001; Ghozland *et al*, 2002; Cheng *et al*, 2004). Thus, conditioned place preference to THC could be revealed in κ knockout mice without avoiding the dysphoric consequences of the first exposure to the drug (Ghozland *et al*, 2002).

The operant drug self-administration paradigm is a predictive and useful animal model to investigate the abuse potential of drugs (Gratton and Wise, 1994; Tanda *et al*, 2000; Vanderschuren and Everitt, 2004; Ahmed and Koob, 2005). Although most drugs abused by humans can be easily self-administered by rodents, attempts to demonstrate operant intravenous self-administration of cannabinoids have been relatively unsuccessful (for a review, see Tanda and Goldberg, 2003). It has been recently reported that synthetic cannabinoid agonists, such as WIN and CP 55,940 maintain self-administration behavior in rats (Fattore *et al*, 2001; Braida *et al*, 2001). However, few studies have revealed the reinforcing effects of WIN in mice by means of a one-session self-administration procedure in restrained animals (Martellotta *et al*, 1998; Ledent *et al*, 1999). Behavioral models of operant intravenous cannabinoid self-administration in freely moving mice are not yet available.

The aim of this study was to establish a reliable model of operant intravenous self-administration of WIN, a potent CB1 cannabinoid receptor agonist (Howlett *et al*, 2002), by avoiding the dysphoric effects of the first exposure to this drug, and to evaluate in this paradigm the possible participation of the κ/dynorphin system in the establishment of such an operant behavior.

MATERIALS AND METHODS

Animals

Male CD1 mice (Charles River, France) and C57BL/6J wild-type and pro-dynorphin knockout mice were used in the present study. The generation of mice with a deletion of the dynorphin gene has been previously described (Zimmer *et al*, 2001). Knockout mice were backcrossed in a pure C57BL/6J genetic background for at least 10 generations. Mice weighed 25–30 g at the beginning of the experiments, and were housed individually in a temperature ($21 \pm 1^{\circ}$C)- and humidity ($55 \pm 10\%$)-controlled room with a 12 h reversed light cycle (lights on between 2000 and 0800 hours). The experiments took place during the dark phase. Water was available *ad libitum* during the self-administration experiments, except during the exposure to the self-administration sessions. Animals were food-deprived 24 h before the first self-administration session and food supply was restricted to reduce the animal's body weight by 10% of their initial weight, thereby facilitating the initiation of self-administration behavior (Fattore *et al*, 2001). Food was given in a single meal immediately after each daily session. All animal care and experimental procedures were conducted according to the guidelines of the European Communities Directive 86/609/EEC regulating animal research, and were approved by the local ethical committee (CEEA-IMAS-UPF).

Drugs

WIN 55,212-2 (Sigma Chemical Co., Madrid, Spain) was dissolved in one drop of Tween 80 and diluted in saline solution. Vehicle also consisted in one drop of Tween diluted in saline solution. Nor-binaltorphimine (Sigma Chemical Co., Madrid, Spain) was dissolved in saline and administered by subcutaneous route (s.c.) at a dose of 5 mg/kg 4 h before the first self-administration session.

Operant Self-Administration Apparatus

The self-administration experiments were conducted in mouse operant chambers (Model ENV-307A-CT, Medical Associates, Georgia, VT, USA) equipped with two holes, one selected as the active hole associated to the delivering of the reinforcer, and the other as the inactive hole. Nose-poking on the active hole resulted in a reinforcer (WIN infusion) or vehicle infusion, while nose-poking on the inactive hole had no consequences. The chambers were housed in sound and light-attenuated boxes equipped with fans to provide ventilation and ambient noise. A stimulus light, located above the active hole, was paired contingently with the delivery of the reinforcer during 3 s, and then left on for an additional period of 3 s.

Surgery for Drug Self-Administration Study

Mice were anesthetized under isoflurane anesthesia (1.5–2.0%), and then implanted with indwelling intravenous (i.v.) silastic catheters, as previously described (Soria *et al*, 2005). Briefly, a 6 cm length of silastic tubing (0.3 mm inner diameter, 0.6 mm outer diameter) (Silastics, Dow Corning, Houdeng-Goegnies, Belgium) was fitted to a 22-gauge steel cannula (Semat, Herts, England) that was bent at a right angle and then embedded in a cement disk (Dentalon Plus, Heraeus Kulzer, Germany) with an underlying nylon mesh. The catheter tubing was inserted 1.3 cm into the right jugular vein and anchored with suture. The remaining tubing ran subcutaneously to the cannula, which exited at the midscapular region. All incisions were sutured and coated with antibiotic ointment (Bactroban, Glaxo-Smith-Kline, Spain). After surgery, animals were allowed to recover for 3 days prior to initiation of self-administration sessions. The catheter was flushed daily with a saline solution in order to maintain its patency. The patency of i.v. catheters was evaluated periodically (approximately every 6 days) and whenever drug self-administration behavior appeared to deviate dramatically from that observed previously, by the infusion of 0.1 ml of thiopental (5 mg/ml) through the catheter. If prominent signs of anesthesia were not apparent within 3 s of the infusion, the animal was removed from the experiment.

Drug Self-Administration Procedure

Mice were trained in the operant chambers to nose-poke for WIN 3 days after surgery. Responding was maintained by

WIN (3.125, 6.25, or 12.5 μg/kg/injection) or vehicle delivered in 35.25 μl over 3 s, through a syringe that was mounted on a microinfusion pump (PHM-100A, Med-Associates, Georgia, VT, USA). The syringe was connected via Tygon tubing (0.96 mm outer diameter, Portex Fine Bore Polythene Tubing, Portex Limited, Kent, England) to a single-channel liquid swivel (375/25, Instech Laboratories, Plymouth Meeting, PA, USA) and to the mouse i.v. catheters. The swivel was mounted on a counterbalanced arm above the operant chamber. Mice were trained to nose-poke in order to receive a WIN injection under an FR1 schedule of reinforcement. Self-administration sessions (2 h daily) were conducted 6 days per week and started with a priming injection of the drug. The house light was on at the beginning of the session for 3 s and off during the remaining duration of the session. A 10 s time-out period was established after each reinforcement. During this 10 s period, the cue light was off and no reward was provided on the active hole. Responses on the inactive hole and all the responses during the 10 s time-out period were also recorded. The session was terminated after 50 reinforcers were delivered or after 2 h, whichever occurred first. The acquisition criteria was defined as (1) 80% of stability in three consecutive sessions, that is, the variance across these 3 days was 20% or less, (2) at least 75% responding on the active hole, and (3) a minimum of three reinforcers per session.

To test the effects of a previous cannabinoid injection on the acquisition of WIN (6.25 and 12.5 μg/kg/injection) self-administration, animals were injected with WIN (0.1 mg/kg i.p.) in the home-cage 24 h before the first self-administration session. Similarly, the effects of a KOR antagonist on the acquisition of WIN (12.5 μg/kg/injection) self-administration were evaluated by injecting nor-binaltorphimine (5 mg/kg s.c.) 4 h before the first self-administration session. The effects of the genetic ablation of pro-dynorphin gene on WIN self-administration were studied in wild-type and pro-dynorphin knockout mice. Both genotypes received a previous injection of WIN (0.1 mg/kg i.p.) in the home-cage 24 h before the first session, and were trained to self-administer decreasing doses of WIN (12.5, 6.25, and 3.125 μg/kg/injection) during 15 days under an FR1 schedule of reinforcement. WIN was available during 5 days at the dose of 12.5 μg/kg/injection, during 6 days at the dose of 6.25 μg/kg/injection, and during 4 days at the dose of 3.125 μg/kg/injection. In a separate experiment, both wild-type and pro-dynorphin knockout mice were trained to self-administer WIN at the dose of 12.5 μg/kg/injection during 10 consecutive sessions. The total intake of WIN for each training dose was calculated considering the number of responses during the last three sessions of the corresponding dose, when all the mice reached the stability criteria. In the case of wild-type mice, an additional training dose of WIN (25 μg/kg/injection) was also evaluated.

Statistical Analysis

Three-way ANOVA, with hole (active or inactive) and treatment (vehicle or WIN; saline or nor-binaltorphimine) or genotype (wild type or knockout) as between-subject factors, and session as within-subject factor, was used to analyze the acquisition of WIN self-administration. Two-way ANOVA with repeated measures (session as within-subject factor and hole as between-subject factor) was used to evaluate the stability for each treatment, when required. Subsequent one-way ANOVAs were performed to compare responding between holes at each session. For the WIN dose-intake curve, values were compared by repeated measures two-way ANOVA (dose as within-subject factor and genotype as between-subject factor). Subsequent one-way ANOVAs were used to calculate differences between genotypes for each dose. In addition, two-way ANOVA with repeated measures (time as within-subject factor and treatment as between-subject factor) was employed to compare responding during the first and second hour of the first self-administration session for each treatment (vehicle or WIN). The χ^2 analysis was employed to compare the percentage of mice that acquire self-administration criteria between the different experimental groups.

RESULTS

Operant Responding for WIN in Mice

CD1 mice were trained to self-administer WIN (12.5 μg/kg/infusion) during 20 days under an FR1 schedule of reinforcement. Operant responding maintained by the cannabinoid was compared with that obtained in a control group trained to self-administer vehicle. As shown in Figure 1, mice were able to acquire an operant responding for WIN only after 2 weeks of training, whereas the animals trained to respond for vehicle did not. Three-way ANOVA revealed a significant main effect of session ($F(19,228) = 6.226$; $p < 0.001$), without effect of treatment ($F(1,12) = 0.114$; NS) or hole ($F(1,12) = 2.670$; NS), and a significant interaction between session and treatment ($F(19,228) = 4.507$; $p < 0.001$). As revealed by subsequent two-way ANOVA, the vehicle group was unable to discriminate between the active and the inactive holes, whereas mice trained to self-administer WIN discriminated between holes after 14 days of training. Thus, a main effect of session was observed for both groups (vehicle: $F(19,76) = 4.758$, $p < 0.001$; WIN: $F(19,152) = 2.131$, $p < 0.01$), and a main effect of hole was detected only in the WIN-treated group (vehicle: $F(1,4) = 0.046$, NS; WIN: $F(1,8) = 5.088$, $p < 0.05$). No interaction between these two factors was observed in either experimental group (vehicle: $F(19,76) = 0.143$, NS; WIN: $F(19,152) = 1.298$, NS). The percentage of animals that reached the acquisition criteria was 60% for mice trained to self-administer WIN (12.5 μg/kg/infusion) and 0% for the vehicle group ($\chi^2 = 5.238$, $p < 0.05$). Interestingly, the mean number of infusions obtained during the first session was significantly lower in mice trained with WIN (12.5 μg/kg/infusion) when compared to the vehicle group (Vehicle: 19.63 ± 4.58; WIN: 12.62 ± 2.42; $F(1,24) = 4.965$; $p < 0.05$).

Effects of a Home-Cage Priming Injection on WIN Self-Administration

To evaluate the possible influence of the aversive effects of the first exposure to cannabinoids on the acquisition of an

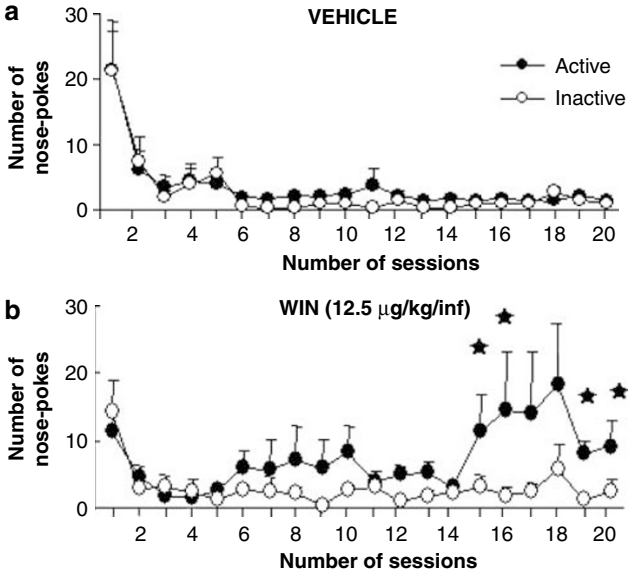

Figure 1 Acquisition of self-administration behavior in mice trained to nose-poke for WIN 55,212-2 (WIN) or vehicle. Number of nose-pokes in the active and inactive holes in 2 h sessions with (a) vehicle and (b) WIN (12.5 μg/kg/infusion) during 20 days. Data are expressed as mean ± SEM ($n = 5$–6 per group). ★$P < 0.05$, comparison between holes (one-way ANOVA).

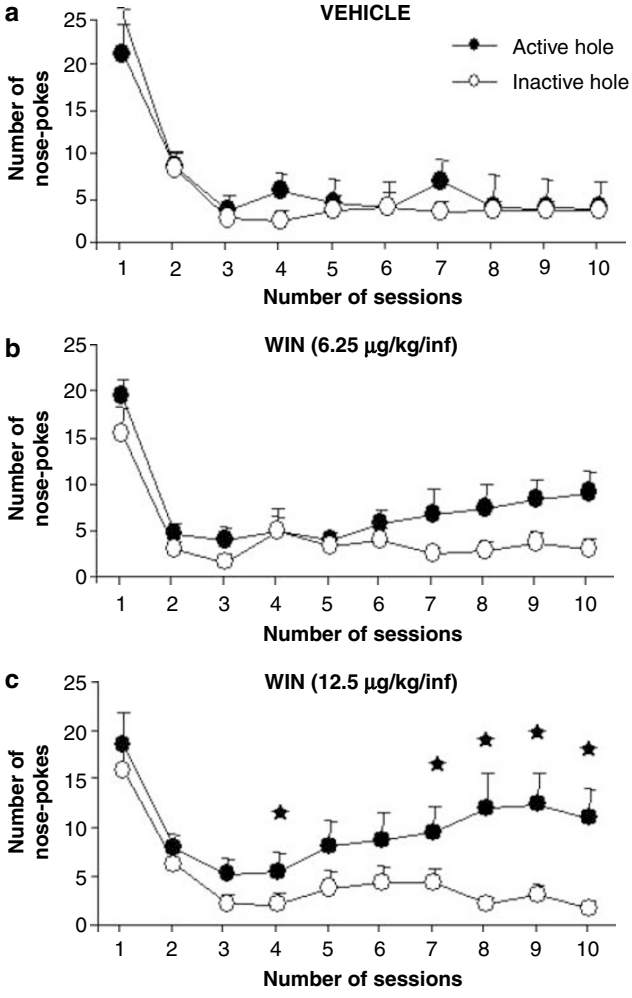

Figure 2 Effects of a priming injection on WIN 55,212-2 (WIN) self-administration. Number of nose-pokes in the active and the inactive holes in 2 h sessions with (a) vehicle, (b) 6.25 μg/kg/infusion, and (c) 12.5 μg/kg/infusion of WIN during 10 days. All the groups were given a previous injection of WIN (0.1 mg/kg i.p.) 24 h before the first session. Data are expressed as mean ± SEM ($n = 6$–8 per group). ★$P < 0.05$, comparison between holes (one-way ANOVA).

operant self-administration behavior, animals received a previous injection of WIN (0.1 mg/kg i.p.) in the home-cage 24 h before the first self-administration session. As shown in Figure 2, CD1 mice trained with WIN (12.5 μg/kg/infusion) discriminated between holes earlier when receiving a previous cannabinoid exposure (Figure 1b). Under these experimental conditions, no significant discrimination was observed in the groups trained with either vehicle or WIN at the dose of 6.25 μg/kg/infusion. Three-way ANOVA revealed significant main effects of the session ($F(9, 306) = 42.71$; $p < 0.001$) and hole ($F(1, 34) = 7.158$; 0.01) without effect of treatment ($F(2, 34) = 0.409$; NS). An interaction between session and treatment was revealed ($F(18, 306) = 1.661$; $p < 0.05$), and no other significant interaction among factors was observed (see Table 1 for two-way ANOVA). The number of infusions obtained during the first session was similar in mice trained with vehicle (22.0 ± 3.63), WIN 6.25 μg/kg/infusion (19.44 ± 1.85), and WIN 12.5 μg/kg/infusion (18.53 ± 3.43). The percentage of mice that reached the acquisition criteria was 0% for the vehicle group, 37.5% for the group trained with 6.25 μg/kg/infusion WIN, and 57.14% for the group trained with 12.5 μg/kg/infusion WIN. Significant differences in the acquisition percentages were found when the vehicle group was compared to mice trained with WIN at the dose of 12.5 μg/kg/infusion ($\chi^2 = 4.95$, $p < 0.05$), but not at the dose of 6.25 μg/kg/infusion ($\chi^2 = 1.98$, NS). No significant differences were observed when comparing percentages of acquisition between the two training doses of WIN ($\chi^2 = 1.60$, NS).

In order to further analyze the different responses of mice receiving or not a previous injection of WIN, we have compared self-administration patterns of WIN (12.5 μg/kg/infusion) during the first session in both groups of animals (Figure 3). When naïve CD1 animals were trained to self-administer WIN (12.5 μg/kg/infusion), the number of infusions during the first hour of the first session

Table 1 Two-Way ANOVA of WIN 55,212-2 Self-Administration Time-Course at Different Training Doses

	Vehicle		6.25 μg/kg/infusion		12.5 μg/kg/infusion	
	F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
Day	$F_{(9.90)} = 24.630$	0.001	$F_{(9.126)} = 22.426$	0.001	$F_{(9.108)} = 6.834$	0.001
Hole	$F_{(1.10)} = 0.003$	NS	$F_{(1.14)} = 3.859$	NS	$F_{(1.12)} = 6.819$	0.05
SxH	$F_{(9.90)} = 0.892$	NS	$F_{(9.126)} = 0.718$	NS	$F_{(9.108)} = 1.163$	NS

Two-way ANOVA repeated measures with hole (H) as between-subject factor and session (S) as within-subject factor. See Materials and methods for details.

was similar in vehicle and WIN groups. However, the number of infusions was significantly reduced during the second hour in WIN-trained animals when compared to the vehicle group. Two-way ANOVA revealed a main effect of time ($F(1,23) = 23.980$; $p < 0.001$) and treatment ($F(1,23) = 4.965$; $p < 0.05$) with no interaction between these two factors ($F(1,23) = 2.675$; NS). Subsequent one-way ANOVA revealed that the effect of treatment was significant within the second hour of the self-administration session ($F(1,24) = 18.334$; $p < 0.001$). Interestingly, this decrease in the number of infusions was prevented by the previous injection of the cannabinoid 24 h before the first WIN self-administration session (Figure 3b). Indeed, a similar number of infusions was observed in vehicle and WIN groups in the two hours of the first self-administration session under these experimental conditions. Thus, two-way ANOVA revealed a main effect of time ($F(1,28) = 47.194$; $p < 0.001$), no effect of treatment ($F(1,28) = 0.517$; NS), and no interaction between these two factors ($F(1,28) = 0.099$; NS).

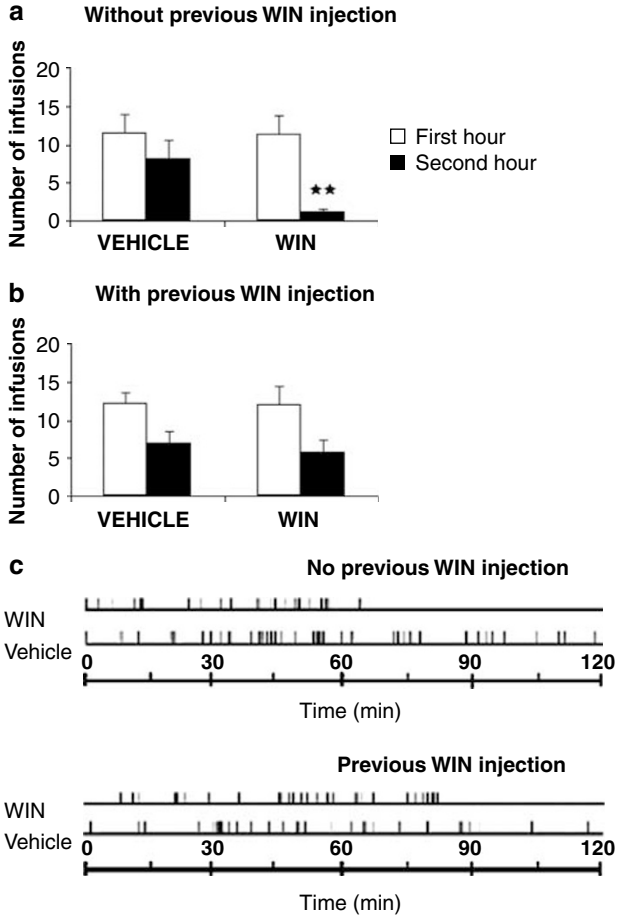

Figure 3 Analysis of pattern and rate of responding during the first self-administration session. Number of infusions obtained during the first and second hour of the 2-h self-administration session (a) without previous treatment and (b) with a previous injection of WIN 55,212-2 (WIN) (0.1 mg/kg i.p.) administered 24 h before. Representative self-administration patterns obtained during the first 2-h session are depicted in (c). Data are expressed as mean ± SEM ($n = 9$–16 per group). ★★$P < 0.01$, comparison between treatments (one-way ANOVA).

Effects of the KOR Antagonist Nor-Binaltorphimine on Cannabinoid Self-Administration

In order to evaluate if the activation of κ/dynorphin system after cannabinoid administration would deter the acquisition of WIN self-administration, naïve CD1 mice were treated with nor-binaltorphimine before the first self-administration training session. As shown in Figure 4, mice pretreated with the κ-opioid antagonist acquired an operant behavior maintained by WIN self-administration on the fourth training session, whereas animals preinjected with saline were unable to achieve this behavior during the training sessions, in agreement with the first experiment. Thus, three-way ANOVA revealed significant main effects of the session ($F(9,216) = 3.350$; $p < 0.01$) and hole ($F(1, 24) = 13.409$; 0.01) without effect of treatment ($F(1,24) = 2.674$; NS). Subsequent two-way ANOVA revealed a main effect of the session that was significant only in the saline group (saline: $F(9, 108) = 2.123$, $p < 0.05$; nor-binaltorfimine: $F(9,108) = 1.567$, NS) and a main effect of hole detected only in the nor-binaltorfimine-treated group (saline: $F(1,12) = 4.327$, NS; nor-binaltorphimine: $F(1,12) = 12.162$, $p < 0.01$). No interactions between these two factors were observed (saline: $F(9,108) = 0.693$, NS; nor-binaltorphimine: $F(9,108) = 1.101$, NS). The percentage of mice reaching the acquisition criteria was signifi-

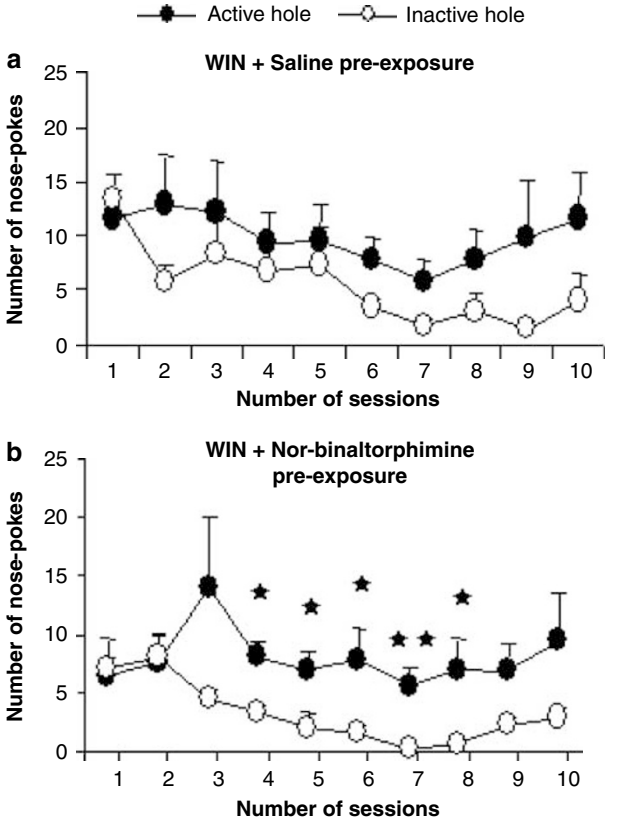

Figure 4 Effects of the KOR antagonist nor-binaltorphimine on the acquisition of WIN 55,212-2 (WIN) self-administration. Number of nose-pokes in the active and inactive holes in 2-h sessions of mice pretreated with (a) saline and (b) nor-binaltorphimine (5 mg/kg s.c.) 4 h before the first self-administration session. Data are expressed as mean ± SEM ($n = 7$ per group). ★$P < 0.05$, ★★$P < 0.01$, comparison between holes (one-way ANOVA).

cantly higher for the group pretreated with nor-binaltorphimine (71.42%) when compared to the saline-treated group (0%) ($\chi^2 = 7.77$, $p < 0.01$).

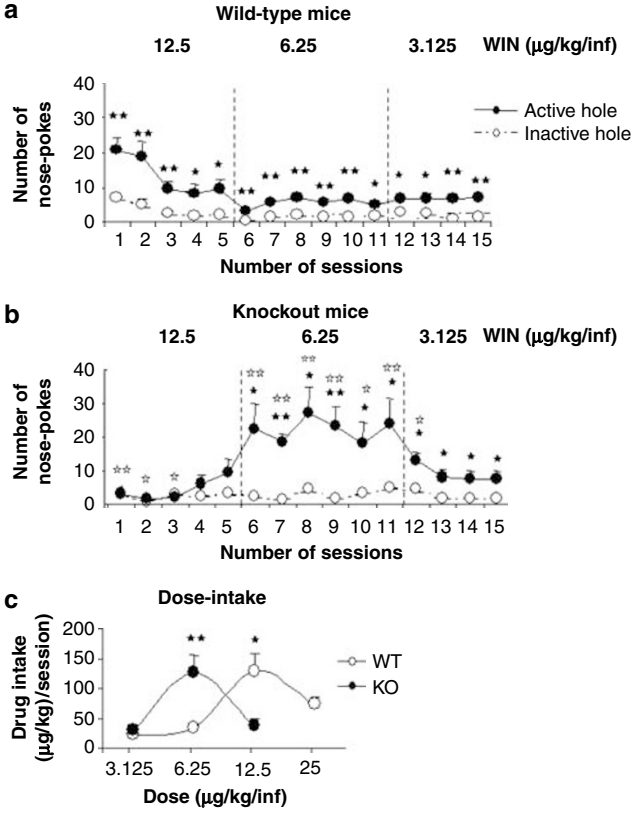

Figure 5 WIN 55,212-2 (WIN) self-administration in wild-type and pro-dynorphin knockout mice. Number of nose-pokes in the active and inactive holes during the 2 h sessions with decreasing doses of WIN (from 12.5–3.125 µg/kg/infusion) during 15 days in (a) wild-type and (b) knockout mice. (c) WIN dose-intake curves in pro-dynorphin knockout and wild-type mice expressed as the total drug intake during the 2-h self-administration session at different doses. Both WT and KO mice were given a previous injection of WIN (0.1 mg/kg i.p.) 24 h before the first session. Data are expressed as mean ± SEM ($n = 6$–8 per group). ★$P < 0.05$, ★★$P < 0.01$, comparison between holes. ☆$P < 0.05$, ☆☆$P < 0.01$, comparison between genotypes (one-way ANOVA).

Self-Administration Maintained by Decreasing Doses of WIN in Wild-Type and Pro-Dynorphin Knockout Mice

Mice lacking the pro-dynorphin gene and wild-type (C57BL/6J) controls received a previous administration of WIN (0.1 mg/kg i.p.) in the home-cage 24 h before the first session, and were then trained to self-administer decreasing doses of WIN (12.5, 6.25, and 3.125 µg/kg/infusion) during 15 days under an FR1 schedule of reinforcement (Figure 5). At the dose of 12.5 µg/kg/infusion, only wild-type animals acquired a stable responding to self-administer WIN (see Table 2 for three-way ANOVA). In addition, the number of infusions in the wild-type animals was significantly higher than in knockouts. Two-way ANOVA revealed that C57BL/6J wild-type mice discriminated between holes at the first self-administration session, whereas knockout animals were unable to discriminate at this dose of WIN during the five training sessions. Thus, a main effect of session was observed in both experimental groups (wild type: $F(4, 80) = 7.653$, $p < 0.001$; knockout: $F(4, 32) = 2.698$, $p < 0.05$), whereas a main effect of hole was only detected in the wild-type group (wild type: $F(1, 20) = 61.949$, $p < 0.001$; knockout: $F(1, 8) = 1.098$, NS). No interaction between these two factors was revealed (wild type: $F(4, 80) = 1.537$, NS; knockout: $F(4, 32) = 1.609$, NS). When the dose of WIN was reduced to 6.25 µg/kg/infusion, knockout mice acquired an operant responding for WIN, and wild-type mice maintained this behavior at a lower rate of infusions (see Table 2 for three-way ANOVA). Both genotypes were able to discriminate between holes throughout the entire period of training with this dose of WIN. Thus, two-way ANOVA revealed a main effect of session only in the wild-type group (wild type: $F(5, 80) = 2.893$, $p < 0.05$; knockout: $F(5, 40) = 1.386$, NS), whereas a main effect of hole was detected in both genotypes (wild type: $F(1, 16) = 19.570$, $p < 0.001$; knockout: $F(1, 8) = 12.147$, $p < 0.01$). No interaction between these two factors was revealed (wild type: $F(5, 80) = 1.027$, NS; knockout: $F(5, 40) = 0.601$, NS). However, the number of infusions obtained during each training session was significantly higher in the knockout than in the wild-type group. At the dose of 3.125 µg/kg/infusion, both genotypes maintained WIN self-administration at a similar rate of responding (see Table 2 for three-way ANOVA). Two-way ANOVA revealed

Table 2 Three-Way ANOVA of WIN 55,212-2 Self-Administration Time-Course at Different Training Doses

	3.125 µg/kg/infusion		6.25 µg/kg/infusion		12.5 µg/kg/infusion	
	F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
Session	$F_{(3.72)} = 8.605$	0.001	$F_{(5.120)} = 2.568$	0.05	$F_{(4.112)} = 2.048$	NS
Hole	$F_{(1.24)} = 25.655$	0.001	$F_{(1.24)} = 30.663$	0.001	$F_{(1.28)} = 33.452$	0.001
Genotype	$F_{(1.24)} = 0.982$	NS	$F_{(1.24)} = 20.351$	0.001	$F_{(1.28)} = 19.469$	0.001
SxH	$F_{(3.72)} = 0.037$	NS	$F_{(5.120)} = 0.757$	NS	$F_{(4.112)} = 0.489$	NS
SxG	$F_{(3.72)} = 6.209$	0.01	$F_{(5.120)} = 2.377$	0.05	$F_{(4.112)} = 5.349$	0.01
HxG	$F_{(1.24)} = 0.431$	NS	$F_{(1.24)} = 14.283$	0.01	$F_{(1.28)} = 5.547$	0.05
SxHxG	$F_{(3.72)} = 2.105$	NS	$F_{(5.120)} = 1.343$	NS	$F_{(4.112)} = 1.568$	NS

Three-way ANOVA repeated measures with hole (H) and genotype (G) as between-subject factors and session (S) as within-subject factor. See Materials and methods for details.

a main effect of session only in the knockout group (wild type: F (3, 48) = 0.743, NS; knockout: F (3, 24) = 8.355, $p < 0.01$), whereas a main effect of hole was detected in both genotypes (wild type: F (1, 16) = 14.514, $p < 0.01$; knockout: F (1, 8) = 11.313, $p < 0.01$). No interaction between these two factors was revealed (wild type: F (3, 48) = 1.440, NS; knockout: F (3, 24) = 0.749, NS). The reduction of the training dose from 6.25 to 3.125 μg/kg/infusion resulted in a decrease in the level of responding only in the knockout group. However, the number of infusions obtained by the wild-type animals was decreased when an additional higher dose of WIN (25 μg/kg/infusion) was tested (WIN 3.125 μg/kg/infusion: 6.68 ± 1.27 inf/2h; WIN 25 μg/kg/infusion: 2.89 ± 0.54 inf/2 h, $p < 0.05$).

In order to test the possibility that lowered responses for WIN 12.5 μg/kg/infusion in the knockouts could reflect delayed acquisition, the training period was extended to 10 sessions in both genotypes in an additional experiment. In agreement with the previous experiment, the number of nose-pokes in the active hole was significantly lower in the knockouts when compared to wild-type mice during the first five sessions. In contrast, the number of responses in the active hole did not differ between genotypes from session 6 to session 10 (session 6: WT = 9.13 ± 1.20, KO = 5.33 ± 2.35; session 7: WT = 8.25 ± 0.88, KO = 7.83 ± 3.09; session 8: WT = 6.38 ± 1.79, KO = 6.17 ± 1.30; session 9: WT = 7.38 ± 1.27, KO = 6.0 ± 1.86, and session 10: WT = 9.0 ± 1.78, KO = 6.0 ± 1.97). However, knockout mice were unable to reach the acquisition criteria, whereas 75% of the wild types did ($\chi^2 = 7.875$, $p < 0.01$). It is of interest to note that these C57BL/6J wild-type mice needed less training sessions to reach the acquisition criteria than CD1 mice trained under the same conditions (CD1: 9.5 ± 0.5 sessions; C57BL/6J: 5.4 ± 0.4 sessions, $p < 0.05$). No significant difference in the percentage of mice reaching the acquisition criteria was observed when comparing C57BL/6J (75 %) and CD-1 mice (57.14%; $\chi^2 = 0.535$, NS).

As shown in Figure 5c, a shift to the left in the dose-intake curve was observed in knockout mice when comparing the mean drug intake during the five sessions to that obtained in wild-type animals. Thus, two-way ANOVA revealed a main effect of the training dose (F (2, 26) = 5.372, $p < 0.05$), no effect of genotype (F (1, 13) = 0.073, NS), and a significant interaction between these two factors (F (1, 13) = 22.983, $p < 0.001$). Subsequent one-way ANOVA revealed significant differences at the doses of 6.25 (F (1.15) = 20.128; 0.01) and 12.5 μg/kg/infusion (F (1.14) = 5.374; $p < 0.05$) when comparing both genotypes.

DISCUSSION

This work demonstrates for the first time that a reliable acquisition and maintenance of cannabinoid self-administration can be revealed in freely moving mice. Using pharmacological and genetic tools, we show the involvement of the κ/dynorphin system in the modulation of cannabinoid self-administration, probably through the mediation of its aversive effects. Thus, the time required to acquire a reliable cannabinoid self-administration was significantly reduced by avoiding its dysphoric effects either by receiving a previous injection of the drug or by the pharmacological blockade of KORs before starting the training sessions. In addition, a shift to the left in the dose-intake curve was observed when WIN self-administration was studied in pro-dynorphin knockout mice and compared to wild-type animals.

Most animal studies designed to demonstrate cannabinoid self-administration have been relatively unsuccessful in spite of the clear reinforcing properties of these compounds in humans. However, THC self-administration has recently been demonstrated in monkeys using a dosage and injection speed parameters different to those previously employed (Tanda *et al*, 2000; Justinova *et al*, 2003). Also within the last few years, reinforcing effects of some synthetic CB1 cannabinoid agonists have been reported using self-administration procedures in rodents. Thus, the CB1 cannabinoid agonist WIN was intravenously self-administered at different doses in chronically catheterized rats (Fattore *et al*, 2001), whereas CP 55,940 also maintained intracerebroventricular self-administration in rats (Braida *et al*, 2001). In mice, the synthetic cannabinoid WIN showed reinforcing effects in naïve restrained animals by means of a one-session self-administration procedure (Martellotta *et al*, 1998), which were selectively mediated by CB1 receptors (Ledent *et al*, 1999). In contrast, the present results show that the mean number of infusions obtained during the first session was significantly lower in mice trained with WIN (12.5 μg/kg/infusion) when compared to the vehicle group, and naïve CD1 mice needed as much as 14 daily sessions to acquire WIN self-administration above vehicle levels. This apparent discrepancy could be due to the different experimental conditions. In the previous studies, mice with restrained mobility received WIN (50 and 100 μg/kg/infusion) when a nose-poke response was performed during a single 30-min session. Our conditions consisted of freely moving animals chronically receiving 2 h self-administration sessions of WIN at lower doses (6.25 and 12.5 μg/kg/infusion) during several days. The analgesic effects of cannabinoids (Martin and Lichtman, 1998) could have some influence on the increased number of self-injections observed in these previous studies, since mice were severely restrained for acute intravenous administration through the tail vein.

The first exposure to a drug of abuse has an important relevance for the acquisition and maintenance of operant self-administration behavior in animals. The present findings suggest that the possible dysphoric effects induced by the first exposure to the cannabinoid agonist would have major consequences on the acquisition of cannabinoid self-administration. Indeed, the analysis of the pattern of self-administration during the first session revealed that naïve CD1 mice nose-poking for WIN dramatically reduced their responses during the second hour of this session. This finding could be either interpreted as a consequence of the aversive effects of the first exposure to the cannabinoid, or as the result of a possible hypolocomotor effect. However, this latter possibility is not likely since the total drug intake attained during the first session (0.125 mg/kg) was lower than the ED_{50} (0.3 mg/kg) required to induce hypolocomotion by i.v. WIN administration in mice (Gifford *et al*, 1999).

The cannabinoid agonists WIN and THC can produce rewarding effects in the mouse place-conditioning para-

digm, but only when animals received a previous injection of the drug (Valjent and Maldonado, 2000; Castañé *et al*, 2004). Accordingly, the present results show that WIN self-administration is clearly improved when mice received a previous WIN exposure. Under these conditions, the negative consequences of the first exposure to the drug could not be associated with the contextual cues or forgotten during the successive sessions. Interestingly, the decrease in the number of infusions observed during the second hour of the first self-administration session was prevented by a previous exposure to the cannabinoid. Similarly, WIN self-administration was facilitated by blocking KORs with nor-binaltorphimine before the first self-administration session. This is in agreement with previous studies showing that nor-binaltorphimine reversed KOR-induced attenuation of morphine (Spanagel and Shoaib, 1994) and cocaine self-administration (Glick *et al*, 1995) and also cocaine discriminative effects (Shippenberg *et al*, 2001). Taken together, these findings give further support to the involvement of KORs in the dysphoric effects of cannabinoids, which would deter the acquisition of cannabinoid self-administration behavior in mice. Accordingly, the anxiogenic-like responses induced by CP 55,940 seem to be mediated by the release of dynorphins and the consequent activation of KORs (Marín *et al*, 2003), and nor-binaltorphimine administration prevented the establishment of THC-induced conditioned place aversion (Zimmer *et al*, 2001). Moreover, THC was able to induce conditioned place preference in mice lacking KORs without receiving any previous injection of the drug, whereas THC-induced place aversion was abolished in these mutant mice (Ghozland *et al*, 2002). In addition, the dysphoric effects of cannabinoids were potentiated in genetically modified mice exhibiting higher levels of dynorphin peptides (Cheng *et al*, 2004). Interestingly, a single exposure to THC has been reported to modify synaptic plasticity in brain areas that are involved in reward and learning, suggesting that cannabinoid activation may transiently alter cognitive functions and motivational behaviors (Mato *et al*, 2004). These changes in synaptic plasticity could be involved in the acquisition of WIN self-administration observed after a single pre-exposure to the cannabinoid agonist.

After a previous cannabinoid exposure, both wild-type and pro-dynorphin knockout mice acquired and maintained an operant behavior for self-administering WIN. However, knockout mice showed a shift to the left in the dose-intake curve when compared to wild-type animals. Indeed, an inverted U-shaped dose-intake curve was observed in both groups of animals, but wild-type mice required a higher dose of WIN (12.5 μg/kg/infusion) than knockouts (6.25 μg/kg/infusion) to obtain the optimal rate of responding. Moreover, extending the training time of WIN 12.5 μg/kg/infusion to 10 sessions revealed that the knockouts increased the number of responses in the active hole, but were still unable to reach the acquisition criteria. Therefore, 12.5 μg/kg/infusion is not an appropriate dose for the acquisition of WIN self-administration in these knockout animals, which is consistent with the hypothesis that the activation of KORs may be involved in cannabinoid negative behavioral responses. KORs and their endogenous ligand dynorphins are abundant in the nucleus accumbens (Fallon and Leslie, 1994), a brain region critically involved in the aversive behaviors associated with dynorphin and exogenous KORs. Thus, conditioned place aversion produced by KOR agonists was abolished by the lesion of the nucleus accumbens (Bals-Kubik *et al*, 1993; Shippenberg *et al*, 1993). In addition, KOR agonists inhibit the rewarding effects of other drugs of abuse, such as morphine or cocaine, which is thought to occur mainly via presynaptic KOR modulation of transmitter release. Accordingly, KOR activation attenuates the elevation of dopamine turnover induced by morphine in the limbic forebrain (Narita *et al*, 1993; Schenk *et al*, 1999). Cannabinoid rewarding effects are also related to an enhancement of dopamine release in the nucleus accumbens (Tanda *et al*, 1997), probably produced by a disinhibition of dopaminergic cells through a decrease of GABAergic signaling (Van der Stelt and Di Marzo, 2003). Therefore, the absence of the inhibitory effect of dynorphin on dopamine release could explain the improvement of cannabinoid self-administration in mice lacking the pro-dynorphin gene.

Interestingly, wild-type mice from C57BL/6J background exhibited a quicker discrimination between holes to self-administer WIN than outbred CD-1 mice, when tested under similar experimental conditions (WIN 12.5 μg/kg/infusion during 10 sessions). Although the final percentage of animals reaching the acquisition criteria was similar between both strains (C57BL/6J: 75%; CD-1: 57.14%), the average number of sessions required to reach such an acquisition criteria was significantly lower for C57BL/6J mice (C57BL/6J: 5.4 ± 0.4; CD-1: 9.5 ± 0.5). In agreement with these results, previous studies have shown that C57BL/6J mice acquire an operant behavior to self-administer cocaine more readily (Grahame and Cunningham, 1995) or show a greater rate of intake than other strains (Kuzmin and Johansson, 2000). In addition, C57BL/6J mice showed a better performance in the Morris water maze test of spatial memory when compared to CD-1 mice (Wright *et al*, 2004). Moreover, the facilitatory effect of rimonabant on acetylcholine release was less intense in hippocampal slices from CD-1 mice as compared to similar slices from C57BL/6J (Kathmann *et al*, 2001).

In summary, the present study shows that the cannabinoid agonist WIN can be self-administered in freely moving mice, a behavioral response induced by most drugs of abuse. Our results corroborate the idea that the prevention of cannabinoid dysphoric effects appears to be crucial for the establishment of this operant behavior in mice, as previously demonstrated for the acquisition of conditioned place preference. Furthermore, we demonstrate that the κ/dynorphin system plays a key role in mediating cannabinoid dysphoric effects and therefore modulates in a negative way their rewarding effects, as previously reported for other drugs of abuse. The development of this intravenous self-administration model in mice represents a useful tool to further investigate the brain pathways involved in the rewarding properties of cannabinoids and their possible interactions with other endogenous systems.

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