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ORIGINAL INVESTIGATION

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Effects of Δ^9-THC and WIN-55,212-2 on place preference in the water maze in rats

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Abstract *Rationale:* Cannabinoids such as Δ^9-tetrahydrocannabinol (Δ^9-THC) or WIN-55,212–2 (WIN-2) have psychoactive effects on cognition. As a result, the reinforcing properties of Δ^9-THC or WIN-2 may confound learning and memory tests with false negative results. It therefore seems advisable to assess the reinforcing properties of the drugs in the same behavioural model used for learning experiments. *Objective:* We therefore developed conditioned place preference protocols in the open-field water maze and tested both Δ^9-THC (2 mg/kg) and WIN-2 (1 mg/kg and 3 mg/kg). Given that previous reports on cannabinoids have revealed conflicting data and that this was a novel behavioural test, we also tested the benzodiazepine receptor agonist diazepam (2.5 mg/kg). Some methodical refinements were appropriate in order to determine the behavioural strategy implemented by the animals. *Methods:* All animals were injected intraperitoneally 30 min prior to training/testing. In experiment 1, male hooded Lister rats injected with drug were repeatedly placed on the drug-related platform and subsequently tested for place preference. In experiment 2, rats were trained to swim to the drug platform on drug days and to the vehicle platform on vehicle days. A series of probe trials was introduced to delineate what had been learned. Experiment 3 studied the effect of WIN-2 on spatial learning in the water maze. *Results:* Neither WIN-2 nor Δ^9-THC induced place preference in the water maze. When trained in the swim procedure, however, WIN-2 was neutral, but Δ^9-THC resulted in place aversion. Conversely, diazepam consistently produced place preference in both procedures. WIN-2 (3 mg/kg), however, produced a small learning deficit in the spatial water maze task. *Conclusion:* It appears that the reinforcing properties of Δ^9-THC and WIN-2 in the doses used here are different, despite them both being agonists at cannabinoid receptors within the central nervous system. The fact that Δ^9-THC may be aversively related to a particular context has implications for previous work reporting deficits in spatial learning.

Keywords Cannabinoid · WIN 55,212–2 · Δ^9-Tetrahydrocannabinol (Δ^9-THC) · Diazepam · Place preference · Water maze · Rat

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Introduction

Conditioned place preference is a method used to measure the reinforcing properties of drugs in animals. Although methods and apparatus used may vary in different studies, the underlying basis of the experiment remains the same. Preference for a particular place develops as a result of the association between the environmental cue or context and the stimulus drug or food (Tzschentke 1998; Bardo and Bevins 2000). Place preference is measured by the amount of time spent in the stimulus-paired context, with an increased amount of time representing preference or a reduced amount of time indicating aversion. A number of advantages of the place preference protocol have been noted: (1) sensitivity to low doses of drug; (2) ability to measure preference or aversion; and (3) testing of subjects whilst in a drug-free state (Carr et al. 1989). However, one possible limitation of the protocol is that of controlling for novelty-seeking behaviour, and previous studies suggest that rats prefer a novel context (Parker 1992; Bardo et al. 1993; Klebaur and Bardo 1999). The apparatus most frequently used to study place preference is a two-chambered conditioning box. Subjects have a central compartment with a choice of two compartments on either side, each one containing different cues. Animals are placed in each compartment on alternative days, with one chamber being used when under drug and the other one being used when under vehicle treatment. This procedure leads, over several days, to the association of the two chambers with the respective compound. When tested drug free, animals may prefer/avoid the drug

chamber rather than the vehicle compartment. Apart from poor dose dependency, this procedure only permits between-subject comparisons and thus requires substantial numbers of animals.

Previous studies into the reinforcing properties of both cannabinoids and the anxiolytic drug diazepam have produced conflicting evidence with respect to the generation of place preference. Cannabinoids are well-known recreational drugs, with Δ^9-tetrahydrocannabinol (Δ^9-THC) being the main psychoactive constituent of marijuana. However, the reinforcing properties of cannabinoids are not well understood, with opposing views on their involvement in self-administration and induction of place preference. Some studies have shown that the classical cannabinoid receptor agonist Δ^9-THC can produce place preference (Lepore et al. 1995; Valjent and Maldonado 2000), whilst others reported place aversion (Lepore et al. 1995; Sañudo-Peña et al. 1997; Mallet and Beninger 1998a; Cheer et al. 2000) or no effect (Sañudo-Peña et al. 1997). The discrepancies in results have been interpreted as being due to differences in apparatus, experimental design and subjects used. Several other synthetic cannabinoid compounds have been developed and their pharmacological and binding characteristics described (Pertwee 1999). Amongst them is the synthetic aminoalkylindole cannabinoid WIN, 55212–2 (WIN-2). WIN-2 shows great similarity in its actions with Δ^9-THC, but consistently induces place aversion (Chaperon et al. 1998). Two other agonists, HU210 and CP 55,940, have been shown to produce place aversion (McGregor et al. 1996; Cheer et al. 2000) or place preference (Braida et al. 2001), whereas the antagonist SR141716A either produced place preference (Sañudo-Peña et al. 1997; Cheer et al. 2000) or had no effect (Chaperon et al. 1998). However, when cannabinoids were administered together with SR141716A, this caused place preference in conditions when cannabinoids alone had no effect (Chaperon et al. 1998). The endogenous cannabinoid receptor ligand anandamide produced no place preference or aversion (Mallet and Beninger 1998a).

By contrast, benzodiazepines are not as common as cannabinoids as recreational drugs. Rather than taken alone, they are normally used in combination with other compounds – such as opioids – and diazepam, a benzodiazepine receptor agonist, is reported to be one of the most frequently used (Barnas et al. 1992). Diazepam alone has been previously studied in place preference protocols and either induces place preference (Spyraki and Fibiger. 1988; Acquas et al. 1989; Borsini et al. 1993; Gray et al. 1999) or has no effect (Meririnne et al. 1999; Leri and Franklin 2000; Matsuzawa et al. 2000). Induction of place preference is in sharp contrast to the spatial learning impairments in the water maze (Zanotti et al. 1994).

This raises the question why psychoactive compounds that induce place preference in one task lead to impairments of spatial learning in another? Or, as observed in some cases, why induction of place aversion could directly account for spatial learning deficits. Such an explanation negates previous behavioural work conducted on spatial paradigms, as the learning deficit may not be related to learning at all, but rather to the dysphoric properties of the drug. A further complication arises from the fact that place preference is usually tested in a two-chamber paradigm, while spatial learning uses open fields or various sorts of complex mazes, thus not permitting direct comparisons of results. We therefore reasoned that establishment of a place preference protocol in a spatial learning apparatus, such as the water maze or radial maze, would greatly facilitate a direct comparison of place preference and spatial learning data and enable a more detailed analysis of the underlying psychopharmacological mechanisms. We here report on a novel place preference procedure in the water maze using WIN-2, Δ^9-THC, and diazepam as test drugs. In a subsequent series reminiscent of a drug discrimination procedure, we developed the test further such that the learning strategy of the animals became apparent. Finally, we conducted a traditional learning experiment using WIN-2 to establish drug-induced spatial learning deficits.

Materials and methods

Experiment 1. Placement-induced place preference in the water maze

Subjects

Twenty-four male hooded Lister rats (Rowett Research Institute, Aberdeen) weighing 250–300 g at the start of training were group housed (four per cage) with free access to food and water on a 12-h/12-h day/night cycle. Experiments were conducted for 16 consecutive days between 1000 hours and 1500 hours. All experiments were performed under UK Home Office regulations (Project License PPl 60/2565).

Drugs

Animals were assigned to three different drug groups (n=8 each): (1) WIN-2 (3 mg/kg, Tocris Bristol UK); (2) Δ^9-THC (2 mg/kg, Sigma UK); and (3) diazepam (2.5 mg/kg, Sigma). Tween 80 was administered as a vehicle on alternate days. All drugs were infused intraperitoneally (i.p.) at an injection volume of 0.5 ml/100 g body weight and 30 min prior to training. Rather than establishing full dose–response relationships, doses were selected on the basis of their efficiency in tests of spatial learning (Δ^9-THC: Heyser et al. 1993; WIN-2: Lichtman et al. 1995; diazepam: Zanotti et al. 1994) and/or place preference paradigms (Δ^9-THC: Lepore et al. 1995; WIN-2: Chaperon et al. 1998; diazepam: Gray et al. 1999).

Apparatus

A circular white Perspex water maze (150-cm diameter, 50-cm depth) was positioned in a room surrounded by various distal (spatial) cues. The pool was filled with water (25±2°C) to a depth of 35 cm. A clear Perspex platform (10-cm diameter) was positioned in the pool at a predetermined site approximately 1 cm below the water surface. All trials were recorded by an overhead video camera and tracker, with data stored both on video and online using a PC-based software (HVS-Image, Hampton, UK) for later analysis.

Training

Before training commenced, all subjects were given a swim trial of 60 s with no platform present. The amount of time spent in each quadrant was measured to determine any pre-training bias for a particular area of the pool. Depending on the preferred quadrant, for example Northeast, the opposing two quadrants (Northwest and Southeast) were selected for training. The design was fully counterbalanced.

An outline of the training protocol is summarised in Fig. 1A. Subjects were injected with either drug or Tween 80 and then placed on the submerged platform for 4 min before being removed and returned to their home cages. If a rat jumped off the platform during the 4 min, it was repositioned on the platform. The following day the subject was given the alternative injection and placed on the platform opposite to the previous day's platform position. This training regime continued for eight consecutive days. On the 9th day, the subjects performed a probe trial. The platform was removed from the pool and animals were released from one of the two non-experimental quadrants and swam freely for 60 s in a drug-free state (probe 1). Then, rats were injected with drug and a second probe trial was given 30 min later (probe 2). Testing under drug was used to determine any state dependency of the association between place and drug (Overton 1984). The time spent in each quadrant was recorded to determine whether place preference was evident. A significantly greater amount of time spent in the 'drug-trained' quadrant was indicative of place preference. The placement training procedure was continued for a further 6 days of alternate drug and vehicle treatment. On the final day, two further probe trials in the absence (probe 3) and presence (probe 4) of drug were given. Again the amount of time spent in each quadrant was recorded.

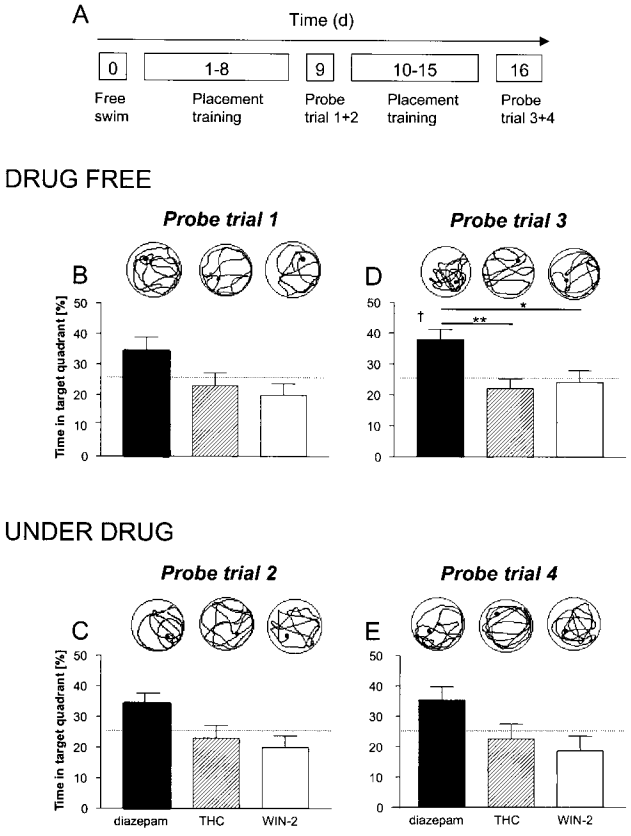

Fig. 1A–E Placement-induced place preference. Means+SEM. *P<0.05, **P<0.01; †P<0.05 compared with chance. **A** Time course of the experiment over 16 consecutive days. **B** There was no difference in time spent in the four quadrants in probe trial 1. **C** Probe trial 2 conducted 30 min post-drug treatment also revealed no group differences for the drug quadrant. **D** Drug groups perform differently during probe trial 3. Animals of the diazepam group spent more time in the drug quadrant than the cannabinoid groups. This place preference was significantly above chance. **E** Probe trial 4 conducted 30 min post-drug injection revealed again that diazepam induces place preference, but Δ^9-THC and WIN-2 had no effect

Experiment 2A. Swimming-induced place preference in the water maze: acquisition

Subjects

Eight male hooded Lister rats (Rowett Research Institute) were used in this within-subject design. Subjects weighed 250–300 g at the start of training and were housed four per cage and subjected to a 12-h/12-h day/night cycle with food and water ad libitum. Animals were trained for 5 days a week (Monday–Friday) between 1000 hours and 1500 hours.

Drugs

WIN-2 (1 mg/kg) and the vehicle Tween 80 were injected i.p. 30 min prior to training.

Apparatus

Apart from some minor modifications (see below) the same apparatus was used as in experiment 1.

Habituation

The habituation phase used visual cue cards containing striations of different width (1 cm and 5 cm) in order to train rats to associate the drug with a cue-related platform location (1 cm was associated with Tween 80 and 5 cm with WIN-2 throughout, or vice versa). Curtains were drawn around the pool and positions of the cue cards and platform under drug and vehicle were counterbalanced for all rats. Cue cards were separated by a barrier and were moved between trials. The platform could be to the left or right of the barrier. Rats were trained in pairs with ten consecutive trials per day and released from one of four predetermined release sites (N, S, E and W) facing the wall directly opposite the cue cards. A maximum time allowance of 90 s was given to swim to and climb onto the platform; if rats failed to do this, the experimenter guided them to the platform where they remained for 30 s. This was followed by 30-s inter-trial intervals spent in their cages. Ten days of habituation training were employed with the number of correct responses recorded for each rat. A correct response was defined as entering the correct quadrant and thus locating the platform without swimming in the incorrect quadrant at all (Robinson et al. 2001a).

Training

Following habituation, the subjects were given 20 days of place acquisition using again WIN-2 and Tween 80. Cue cards and barrier were removed, curtains were drawn back and rats were trained to use the distal cues to find the platform location. The platform was positioned in the centre of one quadrant when animals were administered WIN-2 and in the opposite quadrant when Tween 80 was injected. Release sites were the two non-experimental quadrants. Each subject performed ten trials per day with a maximum time of 90 s to locate the platform and 30 s to remain on it. Then, rats were returned to their cages for an inter-trial interval of 30 s. The schedule of WIN-2 and Tween 80 was non-systematic

with no more than two consecutive days under each treatment. The number of correct responses was recorded and 80% correct responses or above over four consecutive days was set as criterion to determine whether the subjects had learnt the association between place and drug. Other parameters included latency to swim to and climb onto the platform as well as swim speed. On the final day, we conducted a drug-free probe trial to establish whether the procedure had resulted in place preference.

Experiment 2B. Swimming-induced place preference in the water maze: testing

This part immediately followed the acquisition of the swimming task, and both animals and apparatus were identical.

Testing and drug groups

Subjects were well trained at this stage. A series of probe trials combined with training trials were given. On each day, probe trials involved the rat being released from one of two possible release sites and allowed to swim freely in the pool with the platform removed for 60 s before being returned to their home cages. Probe trial 1 of each day was performed drug free. Then drug or vehicle was infused and a post-drug probe trial 2 was administered 30 min later. Rats were then trained in pairs for eight consecutive swim trials. The number of correct responses was recorded for each subject (not shown). A third probe trial was given immediately following the 8th swim trial to determine place discrimination after training.

This protocol was performed using WIN-2 (1 mg/kg i.p. 30 min pre-testing) for 5 days, the dose of WIN-2 was then increased (3 mg/kg) and training continued for a further 5 days. After several weeks of washout, the protocol was repeated with diazepam (2.5 mg/kg) and saline for 8 days using the two quadrants not previously tested. Finally, after a further few weeks of washout, Δ^9-THC (2 mg/kg) was infused with Tween 80 as a control for 8 days of training.

Experiment 3. Spatial reference memory training in the water maze

Subjects

Eighteen male Lister Hooded rats (Rowett Research Institute) weighing 250–300 g at the beginning of the experiment were group housed (four per cage) throughout and subjected to a 12-h:12-h day/night cycle with free access to food and water.

Drugs

WIN-2 (3 mg/kg) or Tween 80 (n=9 per group) was injected i.p. at an injection volume of 0.5 ml/100 g body weight 30 min prior to training.

Apparatus

The same water maze was used as in experiment 1.

Training

A standard reference memory procedure was used with the platform remaining in the same position throughout the experiment. Subjects were naive to the water maze procedure prior to training. Training was performed under drug over four consecutive days with four trials per day. The release point (N, E, S, W) was altered from trial to trial. Subjects were released facing the wall of the pool and given a maximum of 90 s to locate the submerged platform, where they remained for 30 s before being removed. An inter-trial interval of 30 s was employed. If the rats failed to locate the platform, the experimenter guided them to it. Performance of the animals was analysed using various parameters, including latency to locate the platform and swim speed for trial 1 and trial 4 each day.

Testing

Following the 4 days of training, the subjects were given probe trials of 60 s with the platform removed. Probe trials were performed 1, 4 and 7 days following acquisition. Subjects performed one probe trial each day and were released directly opposite to the training platform position and allowed to swim for 60 s before being removed. The amount of time spent in the respective quadrants was measured.

Statistical analysis

Analysis of the data was performed using analysis of variance (ANOVA) with days and drugs as factors followed by appropriate post-hoc tests and a significance level set to $P<0.05$.

Results

Experiment 1. Place preference is induced by diazepam in the placement procedure

Following the initial 8 days of training with 4-min placements on the platform, none of the drugs induced place preference as assessed in probe trial 1 (Fig. 1). ANOVA on the time spent in the drug quadrant revealed no significant difference between the drug groups ($F_{2,23}$=1.98; Fig. 1B) and none was above chance (all P values >0.05, t-test). We therefore reasoned that further drug application might bias the animals towards the drug quadrant. Although probe trial 2 did not yield significant effects ($F_{2,23}$=3.4, P=0.052; Fig. 1C), there was a strong trend towards a difference between groups. The failure to induce place preference was further supported by the fact that times spent in the drug quadrants during probe trials were not significantly different from chance (all P values >0.05, t-test). Interestingly, no drug-induced place aversion was obtained.

Further training for 6 days and testing in drug-free probe trial 3 (Fig. 1D) revealed a main effect of drug ($F_{2,23}$=6.4, P=0.007). Animals in the diazepam group spent reliably more time in the drug quadrant than both cannabinoid groups (P values <0.05), but Δ^9-THC and WIN-2 animals did not differ from each other (P=0.8; Tukey's multiple comparison test). Confirmation of place preference was obtained since the time spent in the drug quadrant was significantly above chance for diazepam subjects (df=7, t=3.98; P=0.005), but not Δ^9-THC (t<1) or WIN-2 (t<1) animals. When tested again under drug treatment in probe trial 4, the same picture emerged (Fig. 1E). However, the group comparison marginally failed to attain significance levels ($F_{2,23}$=3.4, P=0.054). Only diazepam animals seemed to prefer the drug quadrant (dt=7, t=2.3, P=0.054). Neither preference nor aversion was seen in cannabinoid groups (t values <1.3).

Experiment 2A. WIN-2 does not induce place preference in the swimming version of the water maze

Experiment 1 clearly suggests that the cannabinoids Δ^9-THC and WIN-2 did not induce place preference. Since this might be due to methodical reasons, such that placement onto and being confined to the platform is not equivalent to exploration of a box as used in standard place preference procedures, we reasoned that swimming to the platform might provide more detailed information about the environment. This could favour the induction of place preference, but may alternatively be seen as a drug discrimination procedure with WIN-2 being associated with one platform location and Tween 80 with the opposite platform position. A swimming procedure is, however, much more complicated and we therefore included a habituation period. During habituation, animals progressively improved swim patterns by reducing the latency to find and climb onto the hidden platform. The number of errors (Fig. 2A; entries into the non-reinforced quadrant) did not differ between drug conditions (F<1), but there was a main effect of day ($F_{4,35}$=7.2, P=0.0002) and no interaction ($F_{4,35}$=1.5, P=0.24). Enhanced performance also was reflected in the latency (Fig. 2B), with a significant reduction over days ($F_{4,35}$=20.2, P<0.0001) but no drug effects (F values <1). Similar results were obtained for swim speed (data not shown). Phase two comprised acquisition training for two opposite platform positions. Curtains were drawn back to reveal distal cues and we continued injection of WIN-2 and Tween 80. During 20 days of training (Fig. 2C), a pronounced improvement in performance over days was established ($F_{9,70}$=7.21, P<0.0001), and animals responded well under both drug conditions. Although the number of correct responses did reach 80%, there was no main effect of drug or interaction (F<1). Analysis of the latency to swim to and climb onto the platform confirmed these data (Fig. 2D) by showing a progressive reduction during the first 5 days in each drug state after which performance had reached floor levels. Only the main effect of days was significant ($F_{9,140}$=23.6, P<0.0001), but there was no difference between Tween 80 and WIN-2 (F values <1).

While these data suggest that rats did learn about their drug state, there was considerable variability in the animals' performance. To determine the causes of this variability, we examined the sequence of drug treatments. In particular, we were interested in the performance of the animals on the Tween-80 day following 2 days of WIN-2 treatment, or vice versa (3rd day change). Also, we looked at the conditions, when the same drug was applied twice (WIN-2 following WIN-2; Tween 80 following Tween 80), or when the drug was alternate on the next day (Tween 80 following 1 day of WIN-2; WIN-2 following 1 day of Tween 80). These conditions were pooled, since no significant difference between the subgroups was found (all P values >0.05, t-test). Figure 2E displays evidence that animals improved their performance relative to the previous day when in the same or

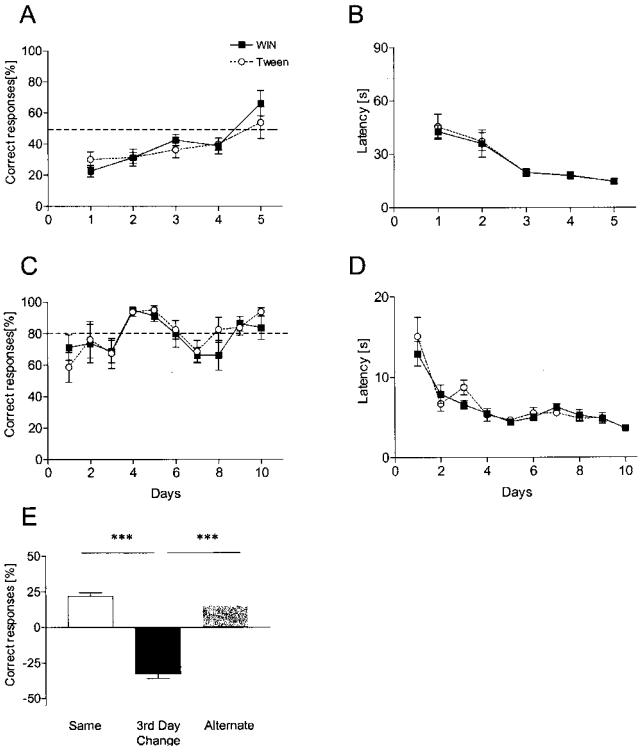

Fig. 2A–E Habituation and acquisition training of place preference in the swimming task using WIN-2 (1 mg/kg) and Tween 80 with ten swim trials per day. Means±SEM; ***P<0.001. **A** During habituation, performance increased over time, although there was no difference between drugs. **B** Latency to locate the platform during habituation was reduced over days. **C** Performance increased with training in both drug conditions and attained 80%. **D** The latency to locate the platform during acquisition decreased with training, although there was no effect of drug. **E** Drugs were infused semi-randomly and thus sequences of WIN-2 and Tween-80 application were sorted according to whether it was the same or an alternate drug on two consecutive days, or whether it was the same for two but different on the third day. Performance improved on the second day of the same or alternate condition, but significantly declined in the 3rd change condition

the alternate condition. By contrast, their performance declined in the 3rd day change condition. This impression was confirmed statistically ($F_{2,149}$=69, P<0.0001) with only the 3rd day change condition being different from both the same and the alternate conditions (t_2>8.7, P values <0.001; see asterisk in Fig. 2B). This suggests that a non-systematic drug treatment is rather confusing to the animals.

Experiment 2B. Testing of place preference in the swimming version of the water maze

Since we gave extensive training under both WIN-2 and Tween 80 and animals showed clear signs of learning, it was important to analyse the strategy employed by the animals. One strategy, for example, could be to always return to yesterday's platform position. Consequently, when the same drug (for example Tween 80) is given, this

would be a correct response, which would be interpreted as drug-induced place preference. When the drug is alternated (for example from Tween 80 yesterday to WIN-2 today), animals employing the yesterday's platform strategy would make an error. The overall interpretation of this result would be that animals have not developed drug-induced place preference.

A second possible strategy is a place preference/avoidance response. Such a response strategy would be recorded from animals always swimming to the same platform location, for example that associated with WIN-2. As this is only correct in 50% of the tests, animals are expected to make errors on Tween-80 days only. However, such a strategy would be indicative of a place preference/avoidance response.

Finally, a drug discrimination response would be indicated by correct responses under both drug conditions as the animal is always aware of its drug state and knows the related platform location. Applying a sequence of probe and training trials distinguished these strategies.

On five consecutive days, we alternated WIN-2 (1 mg/kg) and Tween 80 (data not shown), before increasing the dose to 3 mg/kg WIN-2 (Fig. 3B and Fig. 3C). Pooled data of all drug-free probe trials 1 of each day revealed no bias for any quadrant (Fig. 3B) suggesting no place preference induced by WIN-2 (1 mg/kg or 3 mg/kg). This was confirmed statistically (1 mg/kg: $F_{3,152}$=2.5, P=0.063; 3 mg/kg: F<1). We then rearranged the data to evaluate the time spent in yesterday's target quadrant. A bias for yesterday's platform location was revealed in the WIN-2 (1 mg/kg)/Tween 80 (not shown), but not in the WIN-2 (3 mg/kg)/Tween-80 condition (Fig. 3C; all F values <1). A second probe trial conducted after drug infusion also did not reveal a spatial bias in the WIN-2 groups independent of dose (not shown; F<1.2). These data suggest that animals did not employ a drug-dependent discrimination strategy and WIN-2 did not cause place preference either. Probe trials performed on each day post-training revealed a strong bias for the respective training quadrant at both WIN-2 doses (WIN-2 1 mg/kg: $F_{3,152}$=27.7, P<0.0001; 3 mg/kg: $F_{3,152}$=8.61, P<0.0001).

After several weeks of washout, the same animals received diazepam and saline injections on alternating days. The platform locations used were in the previously neutral quadrants. Pooled probe trial data for a drug-free test revealed a strong bias for the diazepam quadrant (Fig. 3D). There was a main effect of quadrant ($F_{3,248}$=23.1, P<0.0001) confirming this impression, and the time spent in the diazepam quadrant was significantly higher (P values <0.001) than adjacent left and saline, but not adjacent right, quadrant. Animals did not return to yesterday's platform location (Fig. 3e; 2×4 factorial ANOVA: quadrant: $F_{3,248}$=23, P<0.0001; drug and interaction: F<1). The same result was obtained for probe trial 2, which was conducted under drug (not shown). Even when injected with saline, animals preferred the diazepam quadrant (F<1 for drug effect or interaction; quadrant: $F_{3,248}$=17.2, P<0.0001). In probe trial 3, applied shortly after the daily training session, diazepam treatment caused a spatial bias reliably different from the bias induced by training under saline (interaction between drug and quadrant: $F_{3,248}$=24.16, P<0.0001; not shown). In line with experiment 1, these data strongly support the notion of diazepam inducing place preference.

A final test used the main psychoactive constituent of *Cannabis sativa*, Δ^9-THC, to determine its effects on place preference (Fig. 3F, G). Interestingly, we obtained

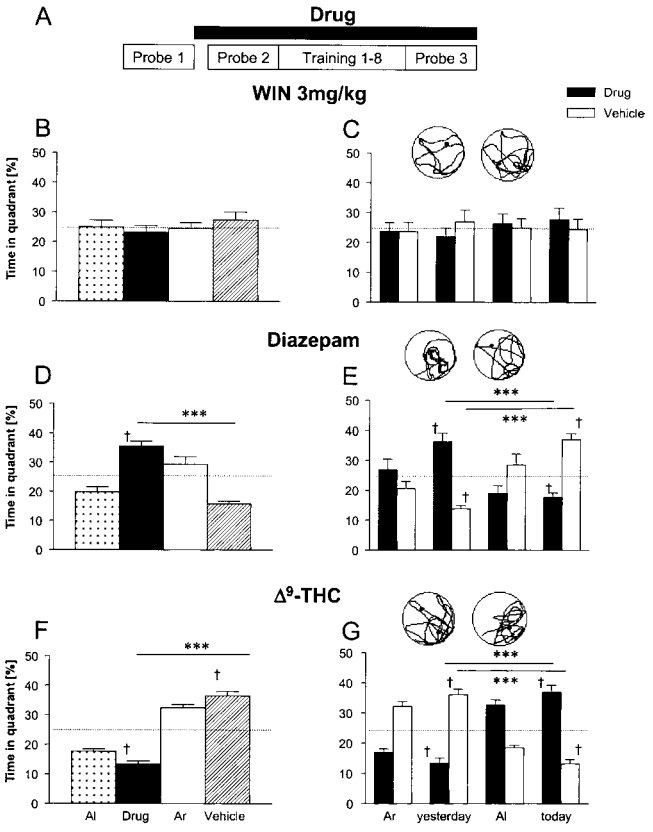

Fig. 3A–G Behavioural strategy employed following administration of WIN-2 (3 mg/kg), diazepam and Δ^9THC. Means+SEM. **P<0.01; †P<0.05 compared with chance. Quadrants: *Al* adjacent left, *Ar* adjacent right with respect to the drug quadrant. **A** Time course of the experiment with all phases under drug except for probe trial 1. Probe trial 1 for WIN-2 (3 mg/kg) revealed no bias for the WIN-2 quadrant (**B**). Probe trial 1 data resorted according to yesterday's platform location (**C**) revealed no preference for yesterday's quadrant. *Filled bars* depict performance when yesterday was a WIN-2 day; *open bars* show performance when yesterday was Tween-80 day. Probe trial 1 for diazepam yielded a strong bias for the diazepam quadrant (place preference: **D**). Resorting for yesterday's platform location showed that animals preferred the diazepam quadrant independent of drug treatment (**E**). *Black bars* represent performance when yesterday was a diazepam day. Animals continuously return to that quadrant. *Open bars* represent performance in probe trial 1 when yesterday was a saline day. Animals prefer today's quadrant (=diazepam quadrant) to yesterday's quadrant. Probe trial 1 for Δ^9THC yielded aversion of the drug quadrant (place aversion: **F**). This was also revealed after resorting (**G**). Animals returned to yesterday's quadrant when yesterday was a Tween-80 day (*open bars*), but preferred today's quadrant, when yesterday was a Δ^9THC day (*filled bars*). Also shown are representative swim traces taken when yesterday was a drug (*left*) or vehicle (*right*) day, respectively

place aversion in this procedure with animals spending significantly less time in the Δ^9-THC quadrant than in the Tween-80 quadrant ($F_{3,252}$=96, P<0.0001; Fig. 3F). Time spent in the Δ^9-THC quadrant was significantly less than in the Tween-80 quadrant (P<0.001; Tukey's multiple comparison), and both values differed reliably from chance (both P values <0.0001, t-test). Again, this became obvious after 2 days of testing with Δ^9-THC. The preference for the Tween-80 quadrant was independent of yesterday's platform location (Fig. 3G). We obtained a drug by quadrant interaction ($F_{3,248}$=95, P<0.0001), which indicates that animals returned to yesterday's platform location when this was a Tween-80 day, but they preferred today's quadrant when yesterday was a Δ^9-THC day. In probe trial 2 (not shown) following drug infusion, rats also spent less time in the Δ^9-THC quadrant, but preferred the opposite Tween-80 quadrant (drug and interaction: F values <1; quadrant: $F_{3,248}$=52; P<0.0001). When tested in probe trial 3, which was conducted after eight training trials, animals responded differently when under Δ^9-THC and Tween 80 (interaction: $F_{3,248}$=38.87, P<0.0001). Animals spent significantly less time in the Δ^9-THC quadrant than in the Tween-80 quadrant (P=0.0002; paired t-test) providing further support for the notion of place aversion induced by Δ^9-THC.

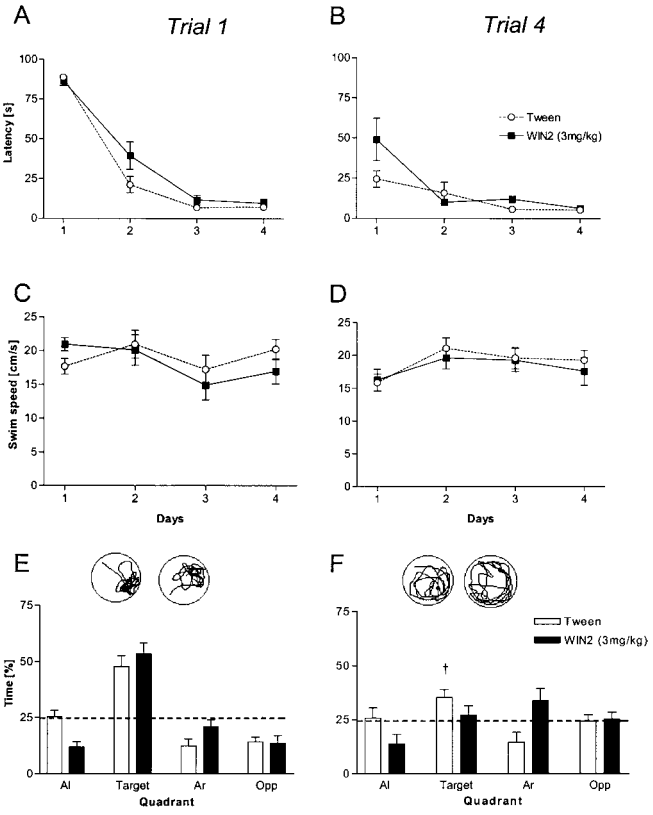

Fig. 4A–F The effect of WIN-2 on a spatial reference memory learning using the water maze. Means±SEM. †P<0.05 compared with chance. **A** Latency to locate platform on trials 1 was increased with WIN-2 relative to Tween. **B** There was no effect of WIN-2 on the latency in trials 4 during acquisition. **C** No effect of drug was observed on the swim speed of trial 1 (**D**) or trial 4 of each day. **E** Probe trial 1 data: amount of time spent in the drug quadrant was similar for both groups (*Al* adjacent left, *Ar* adjacent right). **F** Probe trial 3 conducted 7 days post-training. Tween-80 animals continued to spend a significant amount of time relative to chance in the target quadrant, whereas WIN-2 subjects did not

Experiment 3. Spatial learning in a reference memory task

We set out to identify behavioural elements affected by cannabinoids that may potentially confound spatial memory and thus lead to false negative interpretations of the learning experiments. Towards this end, we obtained place preference for diazepam and a small place aversion effect for Δ^9-THC in experiment 2. WIN-2 used in two different doses, however, proved to be neutral. Thus, the question arises whether WIN-2 may be effective in altering spatial learning when applied both in the same dose and via the same route.

Two groups of animals were trained for 4 days while treated with either Tween 80 or WIN-2 (3 mg/kg) and then exposed to a series of probe trials. Trial 1 of each day (Fig. 4A, Fig. 4C) was treated as a between-session, long-term acquisition memory trial due to a 24-h consolidation period. By contrast, trial 4 reflects within-session learning and thus short-term acquisition memory (Fig. 4B, Fig. 4D, Kesner et al. 1993; Christoffersen et al. 1999). We obtained an overall albeit small acquisition impairment with latencies being prolonged in the WIN-2 group in trial 1 during training ($F_{1,64}$=4.2, P=0.045). Also reliable was the main effect of days ($F_{3,64}$=170, P<0.0001) supporting a performance improvement over days. There was, however, no interaction ($F_{3,64}$=2.4, P>0.05). This long-term acquisition memory deficit was not due to drug-induced alterations in swim speed (Fig. 4C, all F values <2.2, P values >0.05).

In contrast, there was no short-term acquisition memory deficit (Fig. 4B) with latencies to find and escape onto the platform being not different between the two drug groups ($F_{1,64}$=2.7, P=0.1). Although there was a main effect of day ($F_{1,3}$=12.7, P<0.0001), there was no interaction of these two factors ($F_{3,64}$=2.7, P>0.05). Moreover, swim speeds were identical between groups and on the last trial of each day (Fig. 4D, F values <2.5; P values >0.05).

Because the deficit was small, and performance in the WIN-2 group attained floor levels at day 4 of training (Fig. 4A, Fig. 4B), we further examined the strength of the memory trace by employing probe trials 1, 4 and 7 days post-training. A two-way analysis of variance with drug and quadrant as factors revealed a significant interaction ($F_{3,64}$=4, P=0.011) for probe trial 1 (Fig. 4E). This was due to less time spent in the adjacent left quadrant by the WIN-2 group, but the reason is unclear at present. Interestingly, the time spent in the target quadrant did not differ between the two groups. A similar result was obtained for probe trial 2 (not shown). A group difference was revealed at 7 days post-training (Fig. 4F)

for the interaction between drug and quadrant ($F_{3,64}$=5.2, P=0.0027). The time spent in the target quadrant was significantly above chance in the Tween-80 group (t=2.8, df=8, P=0.025), but not in the WIN-2 group (t<1).

Discussion

The water maze as a novel test for place preference

Spatial learning in the water maze requires animals to associate a place with escape from a potentially deadly environment. Similarly, animals exposed to a conditioned place preference/avoidance regime associate the drug with a specific environment. Despite these similarities in associative mechanisms, psychoactive drugs frequently have opposite effects. For example, diazepam in the same dose as used in our study induced place preference (Gray et al. 1999) but impaired spatial learning (Nakamura-Palacios and Roelke 1997). This may suggest two different psychopharmacological effects of diazepam. In the case of Δ^9-THC, however, things are more complicated. Previous work has provided compelling evidence for Δ^9-THC-induced conditioned place aversion (Lepore et al. 1995; Mallet and Beninger 1998a; Valjent and Maldonado 2000) combined with spatial learning deficits (Lichtman et al. 1995). The learning deficit may thus be due to the dysphoric properties of Δ^9-THC and may not be related to cognitive dysfunction. A direct comparison, however, is hampered by the fact that conditioned place aversion and place learning was tested in a different apparatus. To facilitate a comparison, we developed a conditioned place preference/aversion protocol for the open-field water maze. We selected the water maze on the basis that it is frequently used for studies of spatial learning and memory (D'Hooge and De Deyn 2001). Similar to the conditioning chamber, each water maze quadrant contains a set of unique distal cues, which can be used to locate the target platform. The water maze allows the use of four possible quadrants as opposed to just two and may therefore enable repeated drug testing in a within-subject design. Since there are no visual restrictions to any part of the maze, subjects can compare cues between both quadrants during both drug states. The placement of the subject onto a platform positioned in one quadrant of the pool under drug and the opposite quadrant under vehicle was similar to the placement stage in the two-chamber paradigm. A possible problem, which we encountered, was that placement times needed restriction to a few minutes (maximum 4 min), since rats tend to swim away from or jump off the platform during longer placements (Harper 2000).

In agreement with previous work (Spyraki and Fibiger 1988; Acquas et al. 1989; Borsini et al 1993; Gray et al.1999), placement of subjects onto the platform induced place preference in diazepam-treated animals. We found no place preference after treatment with WIN-2 and Δ^9-THC, and this contrasts with findings reported for standard place preference tests (Lepore et al. 1995; Mallet and Beninger 1998a; Cheer et al. 2000; Valjent and Maldonado 2000).

Several possible explanations can be put forward for this discrepancy. First, we did not establish drug effects over a broad range of doses. It is therefore possible that lower or higher doses may induce conditioned place preference or aversion. Our dose of 2 mg Δ^9-THC is in agreement with the dose used by others and may, depending on the experimental conditions, lead to place preference, place aversion (Lepore et al. 1995) or have no effect (Mallet and Beninger 1998a). As for WIN-2, doses of 1 mg/kg have been shown to be aversive in place conditioning (Chaperon et al. 1998). Second, we did not pre-inject drug while animals remained in their home cages. This has been noted to prevent the dysphoric properties of Δ^9-THC (Valjent and Maldonado 2000; Ghozland et al. 2002), therefore bringing about the euphoric properties and causing place preference.

Cannabinoids have differential effects on place learning and place conditioning

The involvement of cannabinoid receptors in spatial learning has frequently been demonstrated using various paradigms, and both Δ^9-THC and WIN-2 resulted in deficits in spatial learning (Lichtman et al. 1995; Molina-Holgado et al. 1995; Lichtman and Martin 1996; Mallet and Beninger 1998b; Hampson and Deadwyler 1998, 1999, 2000;Varvel et al. 2001; Da Silva and Takahashi 2002; experiment 3 in this study). If cannabinoids cause spatial learning deficits, then impairments in place preference would be expected. Previous studies, however, have provided conflicting evidence as to the effects of cannabinoids in place preference/aversion paradigms. In our placement protocol, we obtained no effect of Δ^9-THC. At present, we cannot exclude the possibility that further training would have resulted in place preference as reported by others (Lepore et al. 1995; Valjent and Maldonado 2000). This seems unlikely given that less training in experiment 2B caused robust place aversion, in agreement with previous work on other synthetic cannabinoids including CP-55,940 and HU210 (McGregor et al. 1996; Cheer et al. 2000). A dose-related failure, however, also seems unlikely given that others have consistently used 1–8 mg/kg Δ^9-THC and successfully induced conditioned place preference (Lepore et al. 1998) or place aversion (Mallet and Beninger 1998a). When animals are trained in the water maze, Δ^9-THC induces a learning deficit (Robinson et al. 2001c; Da Silva and Takahashi 2002). This, however, may be due to place aversion rather than a genuine memory impairment.

WIN-2, another synthetic cannabinoid can produce place aversion at the same dose as used in this study (Chaperon et al. 1998). Despite extensive training and multiple testing for relevant strategies, we were unable to replicate this finding in our procedures. Given that no place preference or aversion was obtained for WIN-2, we feel confident that WIN-2-induced learning deficits

(Lichtman et al. 1995; Hampson and Deadwyler 1998, 1999, 2000) are genuinely due to deficits in learning and memory formation, especially because they were obtained in the same dose range. This is particularly so for our studies using spatial learning in the water maze (Robinson et al. 2001b and experiment 3 in this study).

Our data also contrast with the use of WIN-2 in self-administration studies which, like place preference, measures reinforcing effects of drugs. WIN-2 induced intravenous self-administration in mice (Martellotta et al. 1998) and rats (Fattore et al. 2001) but not monkeys (Mansbach et al. 1994). The latter observation is in line with our results of both Δ^9-THC and WIN-2 failing to induce place preference and may be explained by the experimental conditions and species/rat strain employed (Mallet and Beninger 1998a; Cheer et al. 2000; Valjent and Moldonado 2000). In a study by Lepore and colleagues, place preference was evident at a dose of 1 mg/kg Δ^9-THC, lower than the dose used in this experiment, and aversion occurred at higher doses of 2 mg/kg and 4 mg/kg when a 24-h rest period was given between training days (Lepore et al. 1995) in order to prevent the dysphoric effects of repeated drug exposure. Dysphoria may interfere with the reinforcing properties of Δ^9-THC (Parker and Gillies 1995; McGregor et al. 1996) thereby preventing place preference. This does, however, not explain why continuous administration (without resting period) of 1 mg/kg Δ^9-THC had no effect, but higher doses induced place preference (Lepore et al. 1995). An alternative explanation suggests that the reinforcing properties of Δ^9-THC and other agonists may have a late onset at which time animals have been returned to there home cages (McGregor et al. 1996). Since we obtained differential effects for the two cannabinoids tested, this could only apply if the onset of WIN-2 was much later than the onset for Δ^9-THC. This seems unlikely, but a possible way of testing would be to engage longer time periods between drug infusion and training. Moreover, given the differences in pharmacological profiles, one would expect WIN-2 to be more effective than Δ^9-THC in our training protocols. This contrasts with our observations. Finally, the differential effects may be based on the high affinity of WIN-2 for a putative novel cannabinoid receptor (Di Marzo et al. 2000; Breivogel et al. 2001; Hájos et al. 2001), which is insensitive to Δ^9-THC (Breivogel et al. 2001). This would suggest that WIN-2-sensitive and Δ^9-THC-sensitive cannabinoid receptors are differentially involved in conditioned place preference/place aversion. Further tests are warranted to confirm this hypothesis.

Cannabinoids and drug discrimination

Since the results for Δ^9-THC and WIN-2 were not congruent with place preference, the question arises whether both cannabinoids induced drug discrimination. Due to their psychoactive effects, both Δ^9-THC and WIN-2 have been successfully used in drug discrimination procedures (Wiley 1999). Interestingly though, training over 91 sessions was required to achieve stimulus control for 3 mg/kg Δ^9-THC (Järbe et al. 2001). Since our training regime was much shorter and not intended to result in drug discrimination, it seems unlikely that stimulus control was achieved in our tasks. This is consistent with our probe trial data, especially in experiment 2B when animals were tested each day given either drug or vehicle. This never resulted in the expected pattern of drug discrimination performance, i.e. spending the majority of time in the vehicle quadrant when injected with vehicle and spending the majority of time in the drug quadrant when under drug. Consequently, we did not conduct tests for cross generalisation. Rather, our data are consistent, at least for Δ^9-THC and diazepam, with place aversion and place preference, respectively. By contrast, WIN-2 had no effect at all.

Place preference with diazepam

The anxiolytic diazepam has previously been tested for its involvement in place preference but also with conflicting results. Diazepam induced place preference in some investigations (Spyraki and Fibiger 1988; Acquas et al. 1989; Borsini et al. 1993; Gray et al. 1999), but had no effect in others (Meririnne et al. 1999; Leri and Franklin 2000; Matsuzawa et al. 2000). Our results from both experiments strongly support the contention that diazepam induces place preference in rats. However, the onset of place preference differed in the two tasks requiring up to 14 training days to induce robust conditioned place preference. This is a more extensive training period than previously found (Gray et al. 1999) and may be due to the short placement periods (4 min) in experiment 1 relative to the 30-min placement used in the two-chamber procedures (Gray et al. 1999; Klebaur and Bardo 1999; Leri and Franklin 2000; Matsuzawa et al. 2000). Overall, the production of place preference with diazepam supports the reinforcing effects of benzodiazepines. Diazepam-induced place preference was not state dependent (Overton 1984) because a spatial bias was measured in the absence (probe trial 1 in experiment 2B) and presence (probe trial 2 in experiment 2B) of the drug. State-dependent alterations in learning are well established for benzodiazepines (File et al. 1993).

WIN-2 impairs spatial learning in the water maze

Since WIN-2 had no effect on place conditioning, a possible explanation would be that we have used the wrong drug doses. Instead of exploring a full dose–response relationship, we returned to our original aim to dissociate place preference/aversion from spatial cognition and exposed animals to a spatial reference memory task in the water maze with a fixed platform location for four consecutive days followed by probe trials.

WIN-2 in a dose of 3 mg/kg resulted in a learning impairment for trial 1, but not at trial 4 of each day. This does reflect a deficit in between session or long-term memory and is similar to what has been reported for glutamate receptor antagonists previously (Kesner et al. 1993; Christoffersen et al. 1999). While the detailed cognitive mechanism affected by WIN-2 remains elusive at this point in time, these data are indicative of a selective effect on the consolidation of spatial reference memory. They contrast with the work of Hampson and Deadwyler (1998, 1999, 2000) who reported strong encoding deficits in WIN-2-treated animals performing a delayed matching-to-sample task. Such an encoding impairment was not obtained in the water maze since animals attained the same level of performance within each session at trial 4. Thus, WIN-2 can affect spatial learning in the water maze at doses that do not induce place preference/aversion.

Collectively, we show here that the water maze can be successfully used to study conditioned place preference and place aversion, and methodical refinements allow establishment of the learning strategy. This may enable more direct comparisons when the same drugs and doses are studied with respect to spatial learning in the same apparatus.

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